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Interaction of dietary bovine ß2-microglobulin with class I MHC and class I-like molecules in humans and mice: A possible immunological mechanism for the association of cows' milk consumption with autoimmune diabetes.

by

Michael Andrew Craig

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The undersigned certify that they have read, and recommend to the Faculty of Grad Studies and Research for acceptance, a thesis entitled **Interaction of dietary bovine ß2-microglobulin with class I MHC and class I-like molecules in humans and mice: A possible immunological mechanism for the association of cow's milk consumption with autoimmune disease** submitted by **Michael Andrew Craig** in partial fufillment of the requirements for the degree of Master of Science.

To my parents for their patience and love,

My friends who helped me realize what is important in life,

And my confidants, with whom I found the art in science.

ABSTRACT

In an attempt to explain the correlation between milk consumption and incidence of IDDM we considered the following hypothesis. Small quantities of ingested Bovine beta 2-microglobulin (Bovß2-M) might enter the bloodstream intact and interact with human immune-regulatory proteins, thereby modulating the host immune response. We found that orally ingested Bovß2-M can pass intact into the intestine and circulation of adult mice. There it can associate with class I MHC heavy chains as well as with other class I-like proteins. The circulatory half life of Bovß2-M was relatively long ($T_{1/2}$ = 30.1 h), and it was deposited to a considerable extent within pancreatic islet cells. We also found that Bovß2-M associates with homologous human molecules *in vitro*, and that Bovß2-M is present in a biologically active form in commercially processed cows' milk. These results provide background and supportive evidence for our hypothesis.

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ABBREVIATIONS AND DEFINITIONS

AA	amino acid
APC	antigen presenting cell
ß2-M	ß2-microglobulin
BME	2-mercaptoethanol
Bovß2-M	bovine ß2-microglobulin
BSA	bovine serum albumin
CPM	count per minute
CTL	cytolytic T cell
DME	Dulbecco's modified eagle's medium
DMSO	dimethyl sulfoxide
EDTA	ethylene-diamine tetra acetic acid
ER	endoplasmic reticulum
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FITC	fluorescence isothiocyanate
HLA	human leukocyte antigen
HSLAS	Health Sciences Laboratory Animal Services
IDDM	insulin dependent diabetes mellitus
kDa	kilo Daltons
mA	milliamperes
MeOH	methanol
MHC	major histocompatibility complex

Nk1	Natural killer cell surface marker #1
NOD/Ltj	non obese diabetic mice from the Leiter colony (Jackson Labs)
NOR/Ltj	non obese resistant mice from the Leiter colony (Jackson Labs)
OVA	chicken ovalbumin
PBL	peripheral blood lymphocytes
PBS	phosphate buffered saline
pI	isoelectric point
RPMI	Rosewell Park Memorial Institute
SDS-PAGE	Sodium Dodecyl Sulfate - PolyAcrylamide Gel Electrophoresis
TAP	Transporter of Antigen Presentation

CHAPTER 1

BACKGROUND AND INTRODUCTION

A. Thesis synopsis

Several studies have shown that between countries, per capita cows' milk consumption is positively correlated with incidence of autoimmune, insulin dependent diabetes mellitus (IDDM). Although this epidemiological data is very striking, a sound immunological explanation for the association is lacking. One theory proposes that cows' milk antigens are specifically cross-reactive with islet beta-cell antigens, but this idea has not been widely accepted. This led us to consider an alternate hypothesis, that whenever an individual (child or adult) consumes dairy products, small quantities of a bovine immune globulin called beta 2-microglobulin (Bovß2-M; abundant in cows' milk) enter the bloodstream intact, and interact with human immune-regulatory proteins (e.g. class I MHC heavy chains), thereby modulating the host immune response to a variety of environmental and/or self antigens. Bovß2-M is a 98 amino acid disulfide-linked acid-resistant protein which, by displacing the endogenous ß2-M, is capable of pairing with mouse class I MHC heavy chains present on tissue culture cell lines, but its' behavior *in vivo* when orally ingested has not been studied. Using immunoprecipitation assays, we found that orally ingested radiolabeled BovB2M fed by voluntary drinking can pass intact and in a biologically active form into the circulation of adult NOD mice, where it can associate with Class I MHC heavy chains (e.g. D^b and K^d) on splenocytes, as well as with other class I-like proteins such as CD1 and FcR II/III present on cells within the intestinal wall. After intravenous injection of radiolabeled Bovß2-M into mice, we found that the circulatory half life of the protein was relatively long ($T_{1/2}$ = 30.1 h), and that Bovß2-M was deposited heavily on vascular endothelium, and to a considerable extent within pancreatic islet cells. Using in vitro assays on cell lines we were able to show that Bovß2-M can also associate with human class I MHC, FcR II/III and CD1d molecules, and that Bovß2-M is present in

a biologically active form in commercially processed cows' milk. These results provide background, supportive evidence for our hypothesis, although conclusive proof will require additional experiments.

B. The biochemistry and biological function of ß2-microglobulin

Introduction

ß2-microglobulin (ß2-M) is a single chain nonglycosylated polypeptide (approx. 11.5 kDa) which is found free in all body fluids of vertebrates (average concentration ≈1µg/ml), as well as in stable non-covalent association with MHC class I and class I-like heavy chains (1). ß2-M is the smallest member of the immunoglobulin gene superfamily, consisting of a single disulfide-linked immunoglobulin domain which has sequence homology (21-27%) to constant region domains of antibody heavy chains, as shown schematically in figure 1.1A (2). There is considerable homology between the ß2-M of different species, up to 70-80% for closely related species such as bovine and human, and 40-50% for less closely related species (e.g. zebra fish and human). The protein sequences of the ß2-M molecules from a variety of species are shown in table 1.1. In all species examined, ß2-M is encoded by a single gene. Allelic polymorphism in the ß2-M gene, which results in amino acid differences between individuals, have been described in a number of species, including mouse, trout, hamster and bovine. However, this polymorphism, which typically consists of single amino acid changes (e.g. D or A at position 85 in mouse), is minimal compared to that seen for MHC class I heavy chains, and polymorphisms in ß2 are not known to account for differences in immune responses. At this time, only one gene and one amino acid sequence have been found for human ß2-M.

Although the sequences of ß2-M from many different species are known thanks to the cloning of the relevant genes, relatively few of the corresponding proteins have been characterized biochemically or biophysically. For the human, bovine, and mouse proteins, basic biochemical properties such as pI and protein conformation have been determined (3-

5). Considering the high sequence homology between the ß2-M proteins of these three species, there are surprising differences in their biochemical properties, as summarized in table 1.2. Interestingly, bovine ß2-M (Bovß2-M) is known to undergo an irreversible modification resulting in SDS reduction stable dimers, trimers, and tetramers, and this self-association occurs in a time and pH dependent manner (4). The modification is slowed but not completely inhibited at a pH identical to the pI of Bovß2-M, indicating that electrostatic interactions are involved at least initially in the aggregation. This phenomena of self-association has not been observed for human ß2-M under similar conditions, and it probably also does not occur with mouse ß2-M. In terms of biological properties, human ß2-M has chemotactic properties for Thy1+ immature lymphoid cells (6), as well as adjuvant properties for inducing cytolytic T cell responses in peptide immunized mice (7).

As described in more detail below, human ß2-M has been shown to accumulate in the circulation of patients with reanal failure. In these patients, altered human ß2-M (often glycated, that is covalently associated with simple sugars such as glucose, a reaction which occurs more often in poorly controlled diabetes) has been found in polymerized and glycated forms in amyloid deposits. The polymerization or fibrilization has become a common and sometimes debilitating chronic condition in long-term dialysis patients resulting in deposits of protein which interefere with the function of various organs (8). ß2-M in these deposits are predominately acidic isoforms (having a more acidic pI), which are formed by ß2-M interacting with advanced glycation end products (9-11). Acidic isoforms have been identified in patients with a variety of conditions including diabetes and renal failure, but they have not been observed in healthy individuals. (10)

Serum levels of endogenous ß2-M as a marker of disease

Extensive studies have described the value of using serum ß2-M concentration as a marker for a range of diseases from hematological malignancies to HIV progression (12,13). The association of high concentrations of free ß2-M with various malignancies is

most likely a result of activation of the lymphopoietic system and/or the release of ß2-M from the cell surface during cell death. It has been noted that interferon-gamma stimulation increases the surface density of ß2-M on lymphoid cells (14). ß2-M serum levels are also useful as an a index of renal function (2,15-18). In healthy individuals there is a rapid turnover of ß2-M in the plasma (19). In rats and humans, the kidney has been shown to be the main site for removal of ß2-M from plasma. Since ß2-M is small, the protein can easily pass through the glomerular membrane(19). The majority of the filtered protein is reabsorbed in the tubules, where it is either degraded or returned to the circulation. Minor decreases in glomerular filtration rate, and/or disturbances in tubular function can result in significant accumulation of ß2-M in the serum, and the higher concentrations presumably favor the formation of amyloid deposits (19).

Association and exchange of ß2-M with class I MHC

As mentioned above, ß2-M binds strongly to Major Histocompatibility Complex (MHC) class I heavy (i.e. alpha) chains, and it forms an integral part of all functional class I alpha/beta heterodimers (figure 1.1B). ß2-M is absolutely required for the intracellular processing and transport to the cell surface of nearly all MHC class I heavy chains (20,21). However, once on the cell surface class I associated ß2-M is known to exchange with free ß2-M present in the plasma (in vivo) (22) or in tissue culture medium (in vitro) (23). Interestingly, both human and bovine ß2-M are known to efficiently exchange with mouse ß2-M on mouse class I MHC heavy chains, and particularly with D^b (24,25). Differences in the affinity of the ß2-M/class I alpha chain interaction arise not only from the properties of the ß2-Ms, but also from the properties of the alpha chains. In vivo studies with ß2-M knock-out mice have shown differences in relative binding of ß2-M between different class I molecules (26). These studies have indicated that in the mouse, different heavy chains have different affinities for endogenous mouse ß2-M, in the relative order $K^b > D^d > D^b > L^d > K^d$. Consistent with these ideas is the much earlier experimental observation that a

significant population of the class I alpha chains which have a relatively 'low affinity' for mouse ß2-M have been shown to be associated in vitro with xenogeneic, exogenous ß2-M from bovine or human serum present in the tissue culture medium (27-29). In general there appears to be an inverse relation between the affinity of mouse class I heavy chains for endogenous ß2-M versus xenogeneic, exogenous ß2-M.

Xenogeneic ß2-M gives rise to new class I epitopes

The association of bovine ß2-M with mouse class I heavy chains is known to generate new epitopes that can be recognized by CTL clones and complement fixing antibodies(23,30). Presumably some of these new epitopes arise from changes in the conformation of the alpha chains/peptide binding groove, especially for the case of the CTL epitopes. Some experiments suggest that the heavy chain epitopes recognized after xenogeneic ß2-M association are located primarily in the alpha 2 region (31), which could potentially affect how the MHC class I/peptide complex is recognized by T Cell receptors on CD8+ cells, as well as alter the ability of T cells to respond to certain peptides. Therefore, ß2-M not only plays a role in the structure of the class I molecule, but also shapes some of it's functionally relevant epitopes.

Peptide loading of class I MHC

The MHC class I molecule's role is to present endogenous cellular peptides to CD8+ T cells. The mechanism by which this peptide loading occurs is now well understood. Peptides are produced by the action of a multi-component proteolytic unit, the proteasome. These peptides are then transported into the endoplasmic reticulum by the Transporters of Antigen Presentation (TAP), where they bind heavy chain/ ß2-M complexes (32). It is also possible for peptides from exogenous sources to bind class I molecules at the cell surface. This has been demonstrated with the loading of synthetic peptide onto antigen presenting cells or target cells in vitro by simply adding exogenous peptide to the culture medium (33,34). The process behind this exogenous peptide loading

is not well understood, but it has been found that the uptake of exogenous peptide is enhanced in the presence of ß2-M in the cell culture media (35-37).

Two models have been proposed to account for this increased peptide binding. The first puts forth the idea that the known exchange between class I associated and free ß2-M may displace the endogenous peptide, or make it less stably bound, allowing exogenous peptide to take its place. This model assumes that the two exchange processes would be cooperative in nature, so that anything which promoted ß2-M exchange (e.g. an increased concentration of free ß2-M) would also promote peptide exchange. The second model suggests that the presence of exogenous ß2-M stabilizes solitary class I alpha chains on the cell surface, resulting in more available peptide binding sites. Most if not all of these 'rescued' alpha chains would likely still have a bound endogenous peptide, so overall there would be more class I molecules available to exchange peptides. The peptide 'exchange' occurs when a d/b heterodimer loses its endogenous peptide momentarily, and then acquires another peptide from the environment. Until recently experiments have not been able to discriminate between either model. However, the most recent evidence points against the cooperative nature of ß2-M /peptide exchange (38), suggesting that the second model is more likely the correct one.

ß2-M has been shown to be an adjuvant for priming cytolytic T cell immunity induced with peptides (7). Rock showed that human ß2-M is a very effective adjuvant for inducing CTL responses in peptide-immunized mice; mice immunized with peptide alone gave no response, whereas those immunized with peptide plus ß2-M showed strong CTL recall responses to the peptide. This is presumably a side effect of ß2-M's ability to increase class I loading of exogenous peptide at the cell surface, by whatever mechanism. With the estimated lower limit for the number of class I molecules needed to trigger a T cell being placed at 400 to 600 per cell, it is conceivable that the number of ß2-M-free class I heavy chains stabilized by a transitory increase in ß2-M (and able to exchange for or if 'peptideless' directly capture exogenous peptide) might be sufficient to activate resting T

cells. Alternately or in addition, the xenogeneic ß2-M might be acting by a different mechanism, such as providing T helper cell epitopes. No matter what the mechanism(s), it is clear that xenogeneic ß2-M can promote CTL memory against an unrelated peptide which is given at the same time.

Association and exchange of ß2-M with class I-like molecules

Association of ß2-M with cell surface proteins is not restricted to the class Ia (i.e. classical class I) MHC molecules, it is also found bound to all class Ib molecules, as well as to a number of class I-like molecules, whose genes lie outside of the MHC complex. Compared to their polymorphic class Ia counterparts, the Ib family of class I molecules are monomorphic or oligomorphic, and their expression is limited to certain tissues (39,40). The class Ib region also appears to have a higher proportion of pseudogenes in relation to class Ia. While the physiological significance of most class Ib molecules is unknown, several studies have shown that some class Ib molecules can present a specific, limited repertoire of antigens(41). Included in this group are the Qa, Qb, thymus leukemia (Tla) and maternal transfer (Mta) antigens. Regardless of sequence variability or variations in glycosylation, all genes in the class Ib group display an intron/exon structure similar to class Ia genes, and all can bind ß2-M to some degree(42).

Outside the MHC complex are a series of genes capable of forming class I-like heavy chains which associate with ß2-M. These include low affinity Fc receptors (CD16/CD23), maternal antibody transfer receptor (FcRn) and the CD1 antigens. Of considerable interest are the latter two, whose functions have recently come under close scrutiny.

The role of FcRn

The FcRn receptor is responsible for the transfer of maternal antibodies to fetal circulation through the placenta pre-partum and through the intestinal enterocyte post-

partum (43-45). The majority of maternal-fetal IgG transport has been shown to occur after the twenty-second week of gestation in humans (46). At this time in the pregnancy the IgG must cross the syncytiotrophoblast and the fetal capillary endothelium. After isolation of a cDNA encoding the putative human FcRn protein (45), mRNA for the receptor was localized by in situ hybridization to the syncytiotrophoblast, where it actively transports maternal IgG via a receptor mediated endocytic mechanism (47). The homologous receptor has been extensively studied in the neonatal rat gut, where it was first noted to bind and transport IgG in a pH dependent manner (43,48). The same receptor was also noted to be present in a sub-apical endosomal compartment in the murine fetal yolk sack (49). In all tissues the receptor binds monomeric IgG with high affinity at acidic pH, but loses its affinity at neutral pH (e.g. pH of blood). This provides the 'pump' which binds maternal IgG in the acidic environment of the proximal intestine and releases it in the blood where the FcRn/ß2-M /IgG complex is less stable (50).

The requirement for a pH gradient for the function of FcRn is circumvented by a two step process in the placenta. First, non-specific pinocytosis of maternal IgG to an acidic endosomal compartment also transports FcRn to an environment where it is able to stably bind the IgG. Second, the FcRn/IgG/ß2-M complex is shuttled in transport vesicles to the basolateral surface of the cells where at physiological pH the IgG is released into the fetal circulation (49). This is an interesting example of a single receptor being used for the same purpose in two very different environments; in the case of the 'less-conducive' placental environment the receptor is able to function specifically by working in concert with another commonplace non-specific cellular process.

The active transport of maternal antibodies across the gut is known to disappear in most mammals by three weeks of age (48). It was thought that at this point in development the role of the FcRn was completed and it was no longer expressed in adult tissue of any kind. However comparisons continue to be made between the features of FcRn and the features of the postulated Fc protective receptor (FcRp), by which IgG selectively avoids

catabolism in the circulation (51). Shared features between FcRn and FcRp transport include IgG saturation, trans-endosomal movement, enhanced Fc binding in an acid environment, and binding to an identical site on the Fc. In addition, the widespread expression in normal adult tissues of a mRNA which hybridizes to the cloned FcRn, whose tissue distribution appears to correspond to diverse known sites of IgG uptake/catabolism, strongly suggests that there might be similar if not identical Fc receptors at work in the neonate and adult (45,52,53). With the use of ß2-M knock out mice it was shown that the same disruption of ß2-M which eliminates FcRn function also disrupts the selective rescue of IgG from catabolism (54). These results show that the FcRn continues to function on into adult life, but only in the presence of ß2-M.

The CD1 family of molecules

The family of CD1 leukocyte cell surface markers were the first of the cell determinant markers to be recognized by a monoclonal antibody (55). The CD1 proteins are structurally related, although distantly, to MHC class I molecules, and all bind ß2-M(56). None of the various members of the CD1 family (isotypes a, b, c and d in humans) are known to be polymorphic, and all are encoded outside the MHC, on chromosome 1 in humans (57). They are expressed on developing cells in the thymus, on antigen presenting cells in various tissues including intestine, and on cytokine activated monocytes (58,59). Based on similarity between the alpha 1 and alpha 2 domains, the five human isotypes of CD1 can be divided into two subclasses;

a) isotypes CD1 a, b, c, e which are predominately found on cytokine activated lymphocytes, or on developing lymphocytes; these do not occur in mice or rats(55,56).

b) isotype CD1d which is expressed on the majority of intestinal epithelial cells, and is widely conserved in that it is found in all species of mammals studied (60,61).

Several peculiar features of the CD1 family have recently been discovered. CD1b molecules have been found to present microbial lipoglycan antigens to a specific subset of CD4-/CD8-/TCRa/ß negative T cells. Recently a similar function has been demonstrated for CD1c, emphasizing the non-classical nature of antigen presentation carried out by this family of molecules (62). The loading of lipoglycan onto CD1b does not require the associated molecules (e.g. TAP) that are necessary for classical class I antigen presentation, indicating a radically different process is involved (63,64). CD1b has been found to be abundantly expressed in the self-healing lesions of leprosy, where Th1 CD4+ T cells predominate, but not in the lepromatous form where TH2 CD4+ cells are found in higher levels. Observations such as these have led to speculation that the CD1 family may affect the subset of cytokines expressed by T cells (63). Of special interest in relation to ß2-M was the observation that CD1d does not require the presence of ß2-M to be expressed at the cell surface (56). This is in direct contrast to the isoforms of the other group (group a above), which all appear to require ß2-M for transport out of the cell.

Intestinal epithelia and associated lymphoid cells

The intestinal epithelia contains distinct populations of intraepithelial lymphocytes (IELs) whose function remains largely unknown (65,66). IELs from human and mouse are enriched in gamma/delta TCR bearing T cells. However, the predominant population of IELs in humans still have alpha/beta TCRs and are CD8+. The high percentage of CD8+/ alpha beta TCR lymphocytes suggests that the development of the TCR on IELs is likely directed by class I or class I-like molecules, such as those described above. The oligoclonal nature of this population of T cells (67) suggests that the IELs may respond to a limited group of antigens presented on a relatively non-polymorphic ligand. This implicates the nonclassical class I-like molecules which are non polymorphic or oligomorphic. TL or CD1 restricted cytolytic killing has been observed with isolated T cell clones (68-70). Cell lines derived from IECs also display CD1 restricted cytolytic killing,

suggesting that CD1 plays a regulatory role in the extrathymic education which occurs in the intestine.

ß2-M knock out mice suffer from a severely compromised immune system. Due to the absence of MHC class I expression, these mice have almost no detectable alpha/beta CD8+ T cells. In contrast, the gamma/delta CD8+ cells develop normally (71). This strongly suggests that these cells do not require classical class I expression for development. The assumption is that the classical restriction (MHC class I/CD8) is replaced by some ß2-M independent CD8 ligand. CD1 in the mouse (analogous to CD1d in the human) seems a likely candidate since this class I-like molecule can apparently be expressed without ß2-M. However it is still unclear if human CD1d is capable of presenting antigen without ß2-M associated (72). The current belief is that normal CD1 antigen presentation is ß2-M dependent, as recognition of this family can be blocked by conformation dependent antibodies which are almost certainly ß2-M dependent. Mouse CD1 requires ß2-M to present antigen to T cells, which supports the ß2-M dependency theory as well (73). This leaves an undeniably large hole in the possible association of CD1d restriction with the development of gamma/delta $CD8^+$ T cells in ß2-M (-/-) mice, one hypothesis, which will be discussed further in chapter 3, is that dietary b2-M may be able to pair with endogenous CD1 in the gut to provide heterodimers for 'educating' some $CD8^+$ T cells in these knock-out animals.

C. Insulin-Dependent Diabetes Mellitus (IDDM)

Introduction

IDDM is a perplexing disease that has mystified clinicians and scientists since the turn of the century. Early on it was understood that the pancreas played a role in the pathophysiology of diabetes, but until the early 1920's when Banting, Best and Collip first purified insulin and began using it therapeutically, it's integral role in IDDM was unappreciated (74). It is now understood that insulin is secreted by ß-cells which are

located in the islet of Langerhans in the pancreas. Type I IDDM is a chronic autoimmune disease that is characterized by an infiltration of mononuclear cells into the islets resulting in destruction of the ß-cells (75). Autoantibodies produced during ß-cell destruction are present years before the first clinical signs of disease (76,77). Insulin is responsible for the maintenance of carbohydrate metabolism through transport of glucose into cells. Uncontrolled diabetes is seen clinically as increased glucose levels in the blood and eventually in the urine as the re absorption threshold of the kidneys is surpassed. With the advent of insulin therapy, deaths due to the metabolic abnormalities associated with diabetes declined; however, patients still are at risk for complications such as renal failure, proliferative retinopathy leading to blindness, and neuropathic syndromes.

NOD and NOR mice as models of IDDM

Non-obese diabetic mice (NOD) are a well accepted animal model of IDDM. NOD mice are an inbred strain developed from non inbred ICR mice (79). The features of the diabetes syndrome in NOD mice are very similar to human Type I IDDM and include hyperglycemia, glycosuria, hypercholesterolemia and ketonuria. Infiltration of leukocytes into the pancreatic islet cells (insulitis) is the predominant histopathological lesion in NOD mice (79). NOD mice develop diabetes after 13 weeks of age, with the incidence in females being approximately 60% higher than in males (78). As in human IDDM, disease progression in the NOD is modulated by environmental factors such as diet. Environmental effects are also behind the wide range of differences in diabetes incidence observed between various colonies of NOD mice around the world.

Non-obese diabetes-resistant mice (NOR) resulted from an isolated genetic contamination of a NOD/Lt colony with an albino C57BL/KsJ strain (79). In terms of MHC haplotype, NOD mice are K^d, D^b, $I\text{-}A^{NOD}$. The resulting strain is insulitis-resistant and diabetes-free and provides an excellent MHC matched control for NOD studies. NOR/Lt mice exhibit the same peripheral T-lymphocyte accumulation and depressed IL-1

secretion by peritoneal macrophages as seen in NOD. Marker analysis of the NOR/Lt genome indicates that 4 chromosomes contain regions derived from C57BL/KsJ mice (chromosomes 2, 4, 11 and 12). The murine ß-2M gene resides in a cross over region of chromosome 2 causing NOD and NOR mice to differ in their ß2-M alleles. Briefly the NOD strain have the "A" allele of murine ß2-M and the NOR the "B" allele. NOD/NOR ß2-M alleles differ in a single D to A amino acid (AA) change at position 85. This non conservative change from a charged AA to uncharged AA has functional implications. That is, the AA change can affect the affinity of certain class I heavy chains for ß2-M *in vitro*. The change in affinity can in turn affect levels of functional folded class I; for example, the murine D^b molecule is stabilized more by the B form than by the A form of mouse ß2-M.

ß2-microglobulin in milk and association of cows' milk consumption with IDDM

Human ß2-M concentration peaks in human colostrum at the time of delivery (81µg/ml) and drops rapidly until 2 weeks post partum when mean values stabilize at 13µg/ml. These levels are much higher than those found in normal adult sera (1.7µg/ml). Fetal sera levels are much higher than adults, with mean values of 7.2 µg/ml. The relative amounts of Bovß2-M in bovine colostrum (150 µg/ml) and cows' milk (30 µg/ml) are higher than seen in humans. In the normal development of mammals, growing infants are weaned from milk consumption as they approach maturity. The immunological effects of continued consumption of high levels of Bovß2-M past weaning have not been explored in cattle or humans.

In 1984 Borch-Johnsen suggested that there was an inverse correlation between incidence of IDDM and duration of breast feeding (80). Further retrospective studies of IDDM patients quickly refuted this observation (81). Now more than ten years later, the controversy over the correlation between breast feeding and early cow's milk consumption with the incidence of IDDM continues. Several recent studies have found a strong correlation with cow's milk consumption but not with duration of breast feeding (82,83).

Based on these correlation's, it has been proposed that cows' milk may contain a triggering agent or co-factor for the development of IDDM, although the nature of this agent remains undetermined.

D. Experimental hypothesis and goals of this thesis

This thesis is focused on the nature of the agent present in cows' milk which might be responsible for the association of cows' milk consumption with autoimmune diabetes in humans.

We hypothesized that orally ingested, biologically active Bovß2-M present in cows' milk might be able to enter the circulation intact, and to interact with mouse or human immune system proteins, thereby modulating the host immune response to a variety of environmental and/or self antigens. We first set out to determine if orally ingested Bovß2-M was able to reach the small intestine of mice intact, and to interact with murine class I and class I-like proteins present there. As a corollary, we wished to determine if orally ingested Bovß2-M would enter the circulation intact and be able to form heterodimers with class I MHC alpha chains in the spleen. Initial experiments using gavage feeding of mice gave inconsistent results, and forced us to develop an alternate approach based on voluntary feeding. Given positive results with these experiments, we wished to determine (in mice) how long Bovß2-M would remain in circulation, and in what organs the Bovß2-M might be preferentially 'deposited'. Although in vitro association of Bovß2-M with mouse class I and class I-like molecules is well described in the literature, to bring it back to the human situation we wished to see if similar associations occurred with human class I and class I-like molecules, and if different haplotypes of human class I a chains might have different affinities for Bovß2-M. Finally, as this small immunoglobulin is known to have adjuvant properties which might contribute to the formation of autoreactive lymphocytes such as those seen in IDDM, it was of special interest to determine if biologically active Bovß2-M remained in commercial milk after pasteurization. All of these experiments

were designed to provide supportive (although not direct) evidence for a potential mechanism by which cows' milk consumption is correlated with a higher incidence of IDDM.

```
                  10        20        30        40          50
                  '         '         '         '           '
Mouse A    IQKTPQIQVYSRHPPENGKPNILNCYVTQFHPPHIEIQMLKNGKKIPKVE
Mouse B    --------------------------------------------------
Rat        ----------------------F-----S-----Q---EL--------NI-
Guinea Pi  VHLA-RV-------A----Q-FI----SG----Q--VEL-------DN--
Rabbit     V-RA-NV-------A------F-----SG----Q-D-EL---V---EN--
Human      --R--K--------A----S-F-----SG---SD--VDL--D-ER-E---
Bovine     --RP-K----------------Y-----YG----Q---DL----E--KS -
Chicken    ADL--KV-----F-ASA-TK-V---FAAG----K-S-TLM-D-VPMEGAQ


                  60        70        80        90          99
                  '         '         '         '           '
Mouse A    MSDMSFSKDWSFYILAHTEFTPTETDTYACRVKHDSMAEPKTVYWDRDM
Mouse B    ------------------------------------A--------------
Rat        ---L-----------------------V-------VTLK-----------
Guinea Pi  ---L------T--L-V-AA---NDS-E-S---S-ITLS---I-K--PNK
Rabbit     Q--L--N------L-V------NNKNE-S-----VTLK--M--K----Y
Human      H--L---------L-YY-------K-E-----N-VTLSQ--I-K-----
Bovine     Q--L---------L-S-A----DSK-E-S-----VTLEQ-RI------L
Chicken    Y-----ND--T-QRLV-AD---SSGS----K-E-ETLKE-QVYK--PEF
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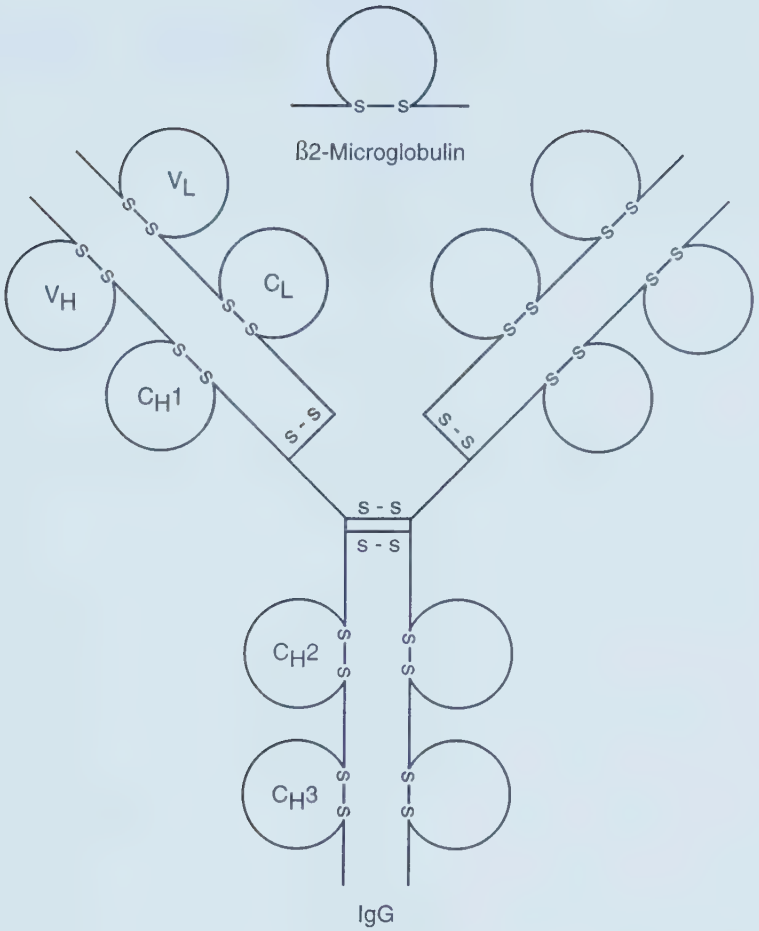
Table 1.1. Amino acid sequences of ß2-microglobulins from a selection of major vertebrate species. Dashes indicate homology to the sequence listed at the top. Conserved cysteines are in bold font (**C**)

Species	Isoelectric point (pI)	Size (total amino acids)	Forms multimers (irreversible)	Solubility at pH 7.4
Human	5.3-5.7	99	No	high
Mouse	7.1	99	No (?)	high
Bovine	6.8	98	Yes	low

Table 1.2. Summary of biochemical properties of ß2-microglobulins from the human, mouse, and bovine species.

A.



B.

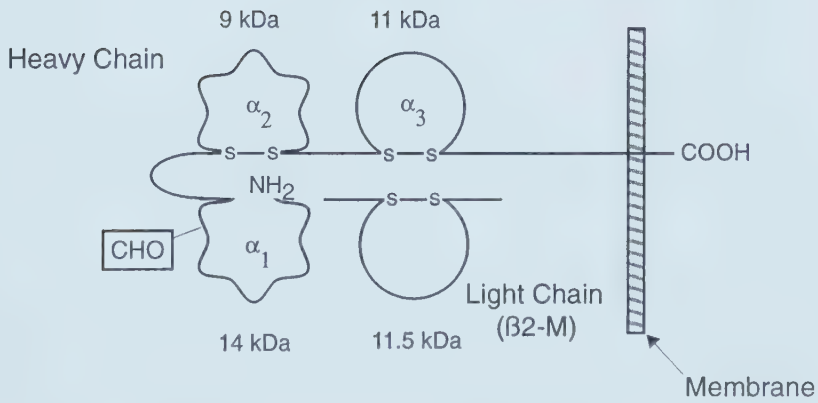


Figure 1.1.

A. Schematic diagram of ß2-Microglogulin in comparison to IgG, in which the heavy chain (H) is divided into four homology regions involving variable (V) and constant (C) domains, and similarly for the light chain (L).

B. Cell surface Class I MHC molecule, comprised of heavy and light chains (i.e. ß2-Microglogulin). Circular elements represent immunoglobulin domains.

CHAPTER 2

MICE FED RADIOLABELED PROTEIN BY GAVAGE SHOW SPORADIC PASSAGE OF LARGE QUANTITIES OF INTACT MATERIAL INTO THE BLOODSTREAM, AN ARTIFACT NOT SEEN WITH VOLUNTARY FEEDING

A. Abstract

Bovine ß2-microglobulin (Bovß2-M) is a small, acid and protease-resistant protein found in cows' milk, which has the capacity to interact specifically with molecules of the mouse immune system. We conducted a series of investigations designed to determine if a portion of orally ingested Bovß2-M could pass intact into the mouse circulatory system. Initial experiments feeding ^{125}I-labeled protein by gavage to fasted mice showed that intact Bovß2-M appeared rapidly and at high levels in the bloodstream (as detected by direct SDS-PAGE analysis of a 1µl sample of plasma followed by phosphorimaging), but this result occurred sporadically and only in about one third of all animals, with no detectable protein appearing in the plasma of the remaining animals. Identical results were obtained when the mice were lightly anesthetized prior to gavage. When a much larger protein, ^{125}I-labeled bovine serum albumin (BSA) was fed by gavage in combination with the labeled Bovß2-M, surprisingly large fragments of the BSA (>50 kDa) appeared in the plasma whenever the Bovß2-M appeared, again in about one-third of the animals. We hypothesized that the passage of relatively large quantities of Bovß2-M into the bloodstream of some mice was due to an intermittent artifact of the gavage feeding (likely spillover into the lungs), and therefore we developed an alternate approach based on voluntary feeding. Mice were easily taught to drink small samples of liquid voluntarily by removing water for 12 h and then manually generating droplets at the end of a 1 ml syringe. When a mixture containing ^{125}I-labeled Bovß2-M and BSA was fed in this manner, no labeled protein could be detected in any animals by direct analysis of a 1 µl sample of

plasma. However, when immunoprecipitation was carried out on a much larger volume of plasma (100 µl), intact Bovß2-M could be found in the bloodstream of all animals, whereas similar immunoprecipitation and analysis for BSA yielded no signal. We conclude that the common method of gavage feeding mice to assess the absorption of orally ingested material can lead to artifacts not seen when the same agent is consumed under more natural circumstances. These observations may have relevance for experiments where oral tolerance is assumed to be induced in rodents by repeated gavage feeding, whereas the same effects cannot be obtained in humans by voluntary feeding.

B. Introduction

Gavage is the traditional method used to deliver small volumes of drugs, etc. into the stomach of laboratory animals for *in vivo* studies. The most commonly used tool for gavage of very small animals such as mice is a syringe attached to a slender curved metal cannulae which has an enlarged, polished spherical end (i.e. gavage needle)(84,85). The gavage tube is placed into the mouth of an immobilized animal and passed gently down the esophagus until the spherical end reaches the cardiac sphincter. Liquid is expelled slowly from the syringe into the distal esophagus, whereupon the animal involuntarily swallows, allowing efficient passage of the liquid into the stomach.

The observations described in the present work originated from experiments designed to test the idea that orally ingested bovine ß-2 microglobulin (Bovß2-M) could pass through the gastrointestinal tract and into the circulation of mice in an intact and biologically active form. The more immunological aspects of these investigations are reported in the next chapter, whereas this chapter focuses on one major methodological aspect, that of the method of feeding. Our studies required that small quantities (≈10 µg) and volumes (≈150 µl) of highly labeled radioactive protein be fed to mice, which allowed for subsequent electrophoretic analysis and quantitation of intact protein as well as potential breakdown products. The use of highly radiolabeled proteins in oral feeding experiments is typically problematic, and in our hands it was impossible to obtain consistent results

utilizing classical gavage feeding. In an attempt to further explore the inconsistency, we developed a second manual feeding method, which allowed us to reliably deliver small volumes of liquid to the animals via voluntary drinking. The data generated afforded us the opportunity to directly compare the voluntary feeding and gavage methods, using precisely the same experimental material in each case. The comparison points out clear limitations to the use of gavage in these types of investigations.

C. Methods

Mice

BALB/c and C57BL/6 mice (female, 8 week), as well as NOD/Ltj and NOR/Ltj mice (male or female, 8-24 week) were obtained from local breeding colonies maintained by the University of Alberta Health Sciences Laboratory Animal Services (HSLAS), or from Jackson Laboratories (Bar Harbor, ME). All procedures involving animals received the approval of the local Health Sciences Animal Welfare Committee, and experiments were carried out under consultation with the veterinary and technical staff of the HSLAS.

Proteins and Radiolabeling

Bovine serum albumin (Fraction V, RIA grade) was purchased from Sigma Chemical Co. (St. Louis, MO), and Bovß2-M was isolated from Bovine colostrum via method by Groves and Greenberg (86). Proteins were labeled by a tyrosine oxidative iodination. Bovß2-M (500 µg) or BSA (500 µg) in 500 µL of PBS pH 5.6 was added to glass tube (12mm x 75 mm Fisher Scientific, U.S.A.) with one IODO-BEAD® per tube (Pierce, Rockford, IL). 5mCi $Na^{125}I$ (IMS.300 Amersham, Arlington Heights, IL) in a vol of 9µL was then added to the solution. Iodination reaction was allowed to proceed at 20°C for 7 min. Reaction was terminated by removing liquid from the glass tube and Iodobead with a pasteur pipette followed by passage through a size exclusion column to separate free ^{125}I from labeled protein. A column consisted of a 10 ml polypropylene

syringe with 5 ml wet volume G-10 sephadex equilibrated in PBS pH 5.6. Fractions (0.5 ml) were collected and 1µL of each fraction counted with a tube gamma counter. Peak fractions were combined and TCA precipitable counts determined as follows. 0.5 ml of 1% BSA (w/v) in PBS pH 5.6 was added to four tubes, and 25 µl pooled fractions added to each tube. To two tubes 0.5 ml of PBS pH 5.6 was added and to the remaining tubes 0.5 ml of 10% TCA in PBS pH 5.6 was added. Tubes were placed on ice for 15 minutes (') and spun at 500 x G at RT for 15'. 20 µl of supernatant from the tubes was removed and counted. Protein associated counts ranged from 95-98% and specific activity was determined by absorbence of solution at OD A280. Bovß2-M specific activity was ≈ $1x10^6$ cpm/µg and BSA specific activity ≈$2x10^6$ cpm /µg.

Gavage feeding

Twelve hours prior to gavage feeding, animals were isolated in metabolic cages without food or water to allow for clearance of the food bolus from the stomach and proximal small bowel. Initial gavage feeding of mice was performed without anesthetic, according to the instructions given by the technical staff of HSLAS. A solution containing ^{125}I-radiolabeled protein (typically 10 µg of Bovß2-M +/- 10 µg BSA, contained in 150 µl of phosphate buffered saline pH 5.6, specific activity ≈1 x 10^6 cpm/µg for each protein) was drawn up into a small gavage needle attached to a 1 ml disposable polypropylene syringe. The gavage needle was as described in the background section, and consisted of a gently curved 20G x 4.5 cm needle with a terminal metal sphere ≈2 mm in diameter. The animals were held vertically, and the gavage needle advanced at an angle (≈30° from vertical) along the tongue and, with the animal swallowing, moved gently down into the esophagus until further advance met slight resistance (typically at a depth of ≈1.2 cm). The protein solution was administered slowly by applying gentle even pressure to the plunger. If resistance was encountered at the plunger the gavage needle was gently withdrawn ≈1-2 mm until the protein solution could be administered by applying the least force. Following

administration of the liquid the gavage needle was gently removed in a single smooth motion. All animals were carefully observed during and for several minutes following gavage, and gavage feeding was deemed to have failed and animals were excluded from the experiment if any liquid appeared in the nasal passages. None of the animals appeared to cough or have difficulty breathing post-gavage, and at post mortem none of the animals showed any sign of stress or injury to the distal esophagus. In an independent experiment a blue vegetable dye solution was fed by gavage using the same method, and none of the dye was seen to have escaped from the esophagus or stomach at post-mortem 30 min later. Due to concerns about possible damage to the esophageal mucosa in animals which struggled during gavage feeding, a second series of gavage feeding experiments were carried out on animals which were lightly sedated with Metophane™ (Janssen Pharmaceuticals, New York, ONT). Anesthetic solution (≈1 ml) was applied to a strip of gauze (≈5 x 25 cm) which had been packed into the bottom of a 10 ml syringe barrel. Mice were made to inhale the vapors from the syringe barrel for ≈1 min or until noticeable relaxation occurred, following which they were immediately fed by gavage as described above. Liquid did not appear in the nasal passages of anesthetized mice any more often than it did in the fully conscious animals, nor did the anesthetized animals appear to have any problems with swallowing. Following gavage animals were returned to the metabolic cage without food or water for 1 h, after which they were transferred to a normal cage and given free access to food and water.

Voluntary feeding

Cages of mice were trained to drink a solution of phosphate buffered saline (PBS) pH 5.6 containing 1% sucrose, from droplets generated manually at the end of 1 ml disposable tuberculin syringe (Becton Dickinson, Franklin Lakes, NJ). This training was accomplished by removing the regular water bottle from the cage for 12 h to create thirst in the animals, and then holding the syringe (with a terminal droplet) in the place where the

water bottle was usually found. Typically after a few minutes one of the animals would discover how to drink from the syringe, and thereafter the others followed very rapidly. Because the animals were unable to actually suck any water from the syringe, the experimenter would have to continually generate fresh droplets at the end of the syringe to satisfy each animal. Several syringes full of liquid were offered sequentially before the animals ceased to drink. The whole water withdrawal/training process was repeated a second time to reinforce the learned behavior and ensure that the mice would rapidly drink the radiolabeled material during the experiment. Mice were isolated in a metabolic cage without food or water for 12 hours prior to feeding them the radiolabeled protein solution. Interestingly, the fasted mice would fail to drink from the syringe as soon as they were placed in a separate cage, so to ensure that each mouse drank their due share of the radiolabeled solution, individual mice were separated from but still in sight of their 'cage-mates' by means of a temporary expanded plastic mesh-work fence which divided the cage in two. Each animal isolated in this manner was allowed to drink from the syringe 500 µl of the PBS/1% sucrose solution containing 1 x 10^7 cpm each of ^{125}I-labeled Bovß2-M and BSA (specific activity ≈1 x 10^6 cpm/µg for each protein). Following drinking each animal was marked and returned to its cage mates, and the subsequent animal isolated by means of the fence, fed, marked, etc. until all animals in the cage had been dealt with. Following voluntary drinking of the radiolabeled proteins the animals were maintained in the metabolic cage without food or water for 1 h, following which they were transferred to a normal cage and given free access to food and water.

Collection of blood and plasma samples

Original studies on gavage fed mice involved taking 20 µL whole blood samples from the tail tip into heparinized glass capillary tubes at various times up to 48h post-gavage. Blood was expelled from the capillaries into 250µl eppendorf tubes with the aid of an adapter attached to a P200 Gilson pipetman. Cells were separated from plasma by

spinning samples in a microfuge at 3000 x G for 30". Plasma was removed from cells, placed in a fresh 250µl tube, and stored at -20°C. Aliquots (1µl) of fresh or frozen plasma were analyzed by SDS-PAGE.

Mice which had consumed the labeled proteins voluntarily were bled as above for 20 µl samples at 4 hours, and then sacrificed at 6 hours post-feeding. Animals were anaesthetized with Metophane™ and bled to termination via cardiac puncture. Whole blood was collected into eppendorf microfuge tubes containing 10% v/v sodium heparin. The blood samples were centrifuged at 4°C for 10 min (500 x G in a microfuge), and serum removed to a fresh eppendorf tube for immunoprecipitation.

Immunoprecipitation

Aliquots of mouse plasma (200 µl) were pre-cleared by mixing with 500 µl of a slurry consisting of 50 mg protein A sepharose 4B-CL (Pharmacia, Mississauga, ONT) per ml of PBS pH 6.8. Tubes were placed on a rotating wheel at 4°C for 12 h, spun briefly in a microfuge, and supernatants removed and divided into two equal aliquots in eppendorf tubes. Two microlitres of the anti-Bovß2-M specific monoclonal antibody CAS-14 (1 mg/ml, grown in bulk culture and purified by protein A sepharose affinity chromatography) was added to one tube, and 2 µl of the anti-BSA monoclonal antibody BSA-33 (1 mg/ml stock, Sigma Chemicals, St. Louis, MO) added to the other, and tubes were rotated at 4°C for 12 h. In preliminary experiments irrelevant isotype matched monoclonal antibodies were also used under identical conditions to control for specificity of the immunoprecipitation. Three hundred microlitres of the protein A sepharose/PBS slurry (as above) was added to each tube, and they were rotated for a further 12 h at 4°C. The tubes were spun briefly in a microfuge, and supernatants removed and stored at -20°C. The sepharose beads were washed two times with 1 ml of wash buffer (0.2% NP40, 150 mM NaCl, 10 mM Tris-HCl pH 6.8) each time, and once with 1 ml PBS pH 6.8. Excess

supernatant was removed and 20 µl SDS loading dye added to the drained sepharose pellets.

SDS-PAGE

All SDS-PAGE gels were cast and run in the BioRad (Hercules, CA) MiniGel™ apparatus, using a 5% Tris/Glycine/acrylamide stacking gel and a 15% separating gel. Samples containing SDS loading dye were heated at 90°C for 5' and loaded immediately onto the gels (5-20µl/lane), which were run at constant current (25 mA) for 50-60 min. Gels were fixed and stained for 15 min in a solution of 50% methanol, 10% acetic acid, 0.5% comassie blue G250; destained for 60 min in 10% methanol, 10% acetic acid; and then dried under vacuum at 80°C for 30 min. Dried gels were placed on a BAS-2Is phosphorimager plate (Fuji, Japan) for 3 days and autoradiographic images captured and displayed using a Fuji phosphorimager and software.

D. Results

Only a sub-set of gavage fed mice showed passage of labeled bovine ß2-microglobulin into the plasma. Figure 2.1 shows representative results obtained when 8 week old female BALB/c mice were fed ^{125}I-labeled Bovß2-M by gavage, and serum samples collected 4 h later and a 1µl aliquot analyzed by SDS-PAGE and phosphorimaging. In this particular experiment, 1 of the 3 mice which were gavaged in a fully conscious state showed labeled protein in the plasma, whereas signal was obtained in 2 of the 3 animals which had been lightly anesthetized prior to gavage. The gavage procedure was identical for each group of three mice (i.e. without or with anesthesia), and there was nothing in any of the animals' behaviors either during or following gavage which would have allowed us to predict which animals would end up with significant radiolabeled protein in their plasma.

Essentially identical experimental results were obtained for a much larger series of animals fed ^{125}I-labeled Bovß2-M by gavage, as summarized in Table 2.1. The animals tested included both male and female, from a variety of strains, and at a variety of ages. Approximately half of the animals were lightly anesthetized prior to gavage. For any individual group of mice, between 25 and 43 percent of the animals showed radiolabeled protein in their serum following gavage. In total, 71 animals were fed by gavage, and 27 of these (38%) showed a clear phosphorimage signal on SDS-PAGE indicating detectable radiolabeled protein in a 1 µl sample of plasma, whereas the remaining animals showed no detectable signal. All animals had clearly ingested the radiolabeled protein, since all were highly radioactive as assessed using a hand-held gamma monitor immediately following gavage. Whether or not the radiolabeled protein made its way to the plasma could not be correlated with the state of immune activation of the animals, level of stress experienced, apparent damage to the distal esophagus at post-mortem, or with the behavior of the animals during or after the gavage procedure.

When a combination of radiolabeled Bovß2-M and BSA were fed by gavage, whenever Bovß2-M appeared in the plasma, very large fragments of BSA also appeared. We conducted a second series of experiments where we fed mice by gavage equal quantities of ^{125}I-labeled Bovß2-M and ^{125}I-labeled BSA and then analyzed 1µl samples of plasma by SDS-PAGE and phosphorimaging as before. Representative results for one such experiment using lightly anesthetized 8 week old female BALB/c and C57BL/6 mice are shown in figure 2.2. Again the passage of Bovß2-M into the plasma appeared to be a sporadic, 'all or none' phenomenon, occurring in 1 of the 3 BALB/c mice, and in 2 of the 3 C57BL/6 mice. Of particular note in these experiments is the fact that in all animals where radiolabeled Bovß2-M appeared in the plasma, additional larger radiolabeled proteins also appeared, which had never been observed when Bovß2-M alone was fed. These larger proteins represent fragments derived from limited proteolysis of the radiolabeled BSA.

When a combination of radiolabeled Bovß2-M and BSA were fed by voluntary drinking, none of the mice showed any radiolabeled protein in the plasma when 1 µl samples were analyzed directly by SDS-PAGE and phosphorimaging. Because of concerns about the sporadic, unpredictable nature of results obtained by gavage feeding, we developed a method whereby mice could be fed small volumes of radiolabeled proteins by voluntary drinking, as described in the materials and methods. Mice were fed in this way the same quantities of ^{125}I-labeled Bovß2-M plus ^{125}I-labeled BSA as had been fed previously by gavage, and after 4 h animals were tail bled and 1µl samples of plasma analyzed by SDS-PAGE and phosphorimaging. In this case none of the 17 animals analyzed (4 NOD/Ltj, 5 NOR/Ltj, 4 BALB/c, 4 C57BL/6; all 8 week old female) showed radiolabeled protein in the plasma (data not shown). This was despite the fact that all animals had clearly ingested the radiolabeled protein, since after feeding all mice were highly radioactive as assessed using a hand-held gamma monitor.

When a combination of radiolabeled Bovß2-M and BSA were fed by voluntary drinking, Bovß2-M but not BSA could be detected in the plasma of all mice by immunoprecipitation of 100µl of serum. The animals fed a mixture of Bovß2-M and BSA by voluntary drinking as described in the previous section were sacrificed after 6 h, and a much larger volume of plasma collected by cardiac bleed. A 200 µl sample of plasma was pre-cleared with protein A sepharose, split into two equal parts, and subjected to immunoprecipitation using Bovß2-M specific or BSA specific monoclonal antibodies in order to concentrate any radiolabeled proteins which had made their way into the plasma. Proteins recovered from the separate immunoprecipitations on each plasma sample were pooled, run on SDS-PAGE, and the gels analyzed using a phosphorimager. A typical autoradiographic image obtained in this series of experiments is shown in figure 2.3, which represents results obtained for 4 separate BALB/c mice. In all mice a clear Bovß2-M band can be seen after immunoprecipitation, whereas no larger molecular weight bands

corresponding to BSA or its proteolytic products are observed. Essentially identical results were obtained for all 17 mice fed the radiolabeled protein mixture by voluntary drinking.

E. Discussion

In conducting investigations to determine if a portion of orally ingested bovine ß2-microglobulin could pass intact into the mouse circulatory system, we first adopted the apparently straightforward method of feeding by gavage ^{125}I-labeled protein, and analyzing 1µl samples of plasma by direct SDS-PAGE and phosphorimaging. Surprisingly, about one third of the fed animals showed a strong signal for the radiolabeled protein in plasma, whereas no signal was detected in the remainder of the mice. In order to further characterize this phenomenon, we conducted a second series of experiments where we fed mice by gavage equal quantities of ^{125}I-labeled Bovß2-M and ^{125}I-labeled BSA.

Although Bovß2-M would be expected to be relatively stable to acid and digestive proteases (i.e. the conditions found in the stomach and small bowel), this would not be the case for BSA (87). Thus we reasoned that if intact BSA or very large fragments derived from it were to appear in the plasma along with Bovß2-M, then the sporadic passage of the radiolabeled protein(s) into the circulation was unlikely to be occurring in the small intestine. Indeed this proved to be the case, and led us to consider as more likely explanations either random breaks in the distal esophageal mucosa, or occasional spillover/regurgitation of radiolabeled protein into the lungs. We favor the latter of these two possibilities, since BSA is known to be stable in mouse plasma, and were it to gain access to the circulation via esophageal lesions we would expect it to be largely undegraded, which is clearly not the case (figure 2.2). Albumin is known to be degraded by pulmonary macrophages and to be poorly absorbed by alveoli (88), whereas ß2-microglobulin (rat or human) is highly stable in the lungs of rats (89), and given its size, would be expected to be more efficiently absorbed than albumin. Since figure 2.2 clearly shows that smaller amounts of partially degraded BSA are absorbed compared to larger

amounts of intact Bovß2-M, these ideas are most consistent with the hypothesis that the lungs are the site of absorption of the radiolabeled proteins in the approximately 1 in 3 animals where relatively large quantities of protein appear in the serum. In addition, measurements of Bovß2-M plasma half life performed on these animals (data not shown) suggests the presence of a reservoir of protein outside of the vascular and extravascular compartments; the lungs, but not the esophagus, would provide such a reservoir.

It is likely that despite technical competence and the best efforts of the investigator, with mice a natural and inevitable result of gavage is occasional regurgitation and aspiration, perhaps while the gavage needle is still in place, thus allowing small quantities of liquid to pass into the lungs. The quantity of liquid involved does no apparent harm to the animals, and there is no practical way to tell in which animals this 'micro-aspiration' has occurred. It is only with an extremely sensitive assay such as the one used in the present investigation that the phenomenon becomes apparent, and indeed we see that it occurs randomly in about one-third of animals. For those who might question our competence with the gavage method, we can only say that we perform the procedure the same way each time, and two-thirds of the time we have no 'micro-aspiration', and therefore demonstrate competence! In fact, in the field of pharmokinetics, even with expert technical staff random gavage related deaths termed 'dosing related deaths' have been well documented, particularly in mice, and these are believed to result from the introduction of hypertonic solutions into the lungs (90).

It is possible that with other methods of gavage, such as the use of rubber canulas which are passed into the stomach, less potential damage to the esophagus is possible however these techniques are more prone to 'micro-aspiration'. Furthermore, such methods represent if anything more severe interventions, and they are notoriously difficult to implement in mice. Given the uncertain benefit and technical difficulty of these methods, we decided to pursue the simple, natural, but unproven method of seeing if we could teach mice to reliably drink small quantities of liquid from a hand-held 1 ml syringe. This proved

to be quite straightforward and once the animals were taught, was actually more efficient than feeding by gavage.

None of the mice fed radiolabeled proteins by the voluntary method showed the relatively high plasma levels of radiolabeled protein which occurred sporadically in about 1 in 3 of the gavage fed animals. We assume this means than none of the voluntary fed animals experienced 'micro-aspiration', and therefore conclude that feeding by voluntary drinking reflects what happens when mice consume liquids under natural circumstances. Indeed these are the only conditions under which we could pursue our original experimental question; that is, we wished to determine if a portion of orally ingested bovine ß2-microglobulin could pass intact into the mouse circulatory system under natural conditions. Given that none of the mice fed by voluntary drinking showed any radiolabeled protein by direct analysis of 1 µl of plasma, we went on to use immunoprecpitation of much larger volumes of serum (100 µl) in order to see if the protein was present at much lower levels, an idea which had not occurred to us while dealing with the very strong signals obtained in the approximately one third of gavage fed animals. The immunoprecipitations clearly demonstrated that small quantities of orally ingested bovine ß2-microglobulin are able to pass into the circulation, and that this phenomenon occurred in all animals tested. This result provides a second positive control (in addition to monitoring the animals with a hand-held gamma counter after voluntary feeding) to demonstrate that all animals actually did consume the radiolabeled ß2-M. In contrast, we were unable to immunoprecipitate full length BSA from 100 µl of plasma, although equal amounts (and counts) of BSA were clearly ingested at the same time as the Bovß2-M. It is possible that relatively large proteolytic fragments of BSA were able to pass into the plasma, but that the anti-BSA monoclonal antibody used in the immunoprecipitations was unable to bind these (although the same monoclonal antibody did give a signal on Western blots, suggesting that it bound to a linear epitope). An alternative explanation, and the one which we favor, is that because all radiolabeled proteins fed by voluntary feeding ended up being absorbed

only in the gastrointestinal tract, the BSA was significantly degraded prior to absorption, and the relatively small fragments generated were not efficiently immunoprecipitated, nor would fragments of this size have been apparent on the SDS-PAGE gels used in these experiments.

Pharmacological studies commonly involve compounds that are not palatable to animals (84,85). Alternatives to gavage feeding for such compounds have been previously described (91). These methods require preparation of binding agents and the mixing of foodstuffs with the compounds being studied. Unfortunately these approaches may be impractical when studying radiolabeled or highly toxic compounds, since the material may be limited, and/or the potential for investigator exposure may be prohibitive. In these cases gavage feeding remains the only method of delivering a low volume bolus of the compound of interest. However, for all compounds which are palatable, even if they are in limited volumes and/or are highly radioactive or toxic, the voluntary liquid feeding method provides a preferable alternative to gavage, due to the ease of sample preparation, minimal investigator exposure time (once animals have been taught to drink), and freedom from artifact. In particular voluntary feeding should be the method of choice when one wishes to determine whether or not orally ingested materials (e.g. radioactively labeled purified proteins or other compounds) are absorbed via the gastrointestinal tract under natural circumstances. Gavaging is an inherently complex technique, the success of which depends on several factors, whereas voluntary feeding is able to avoid virtually all of these difficulties, as summarized in table 2.2.

Several studies have used gavage feeding of antigens to induce oral tolerance, for example, to myelin basic protein (92). Given our results, we would reasonable predict that one in three times, a relatively larger quantity of the fed antigen would be absorbed, likely without having passed through the gastrointestinal tract. The sporadic passage of this relatively larger quantity of antigen directly into the bloodstream (probably via the lungs) may have specific effects on the immune system which would not occur were the same

antigen to be fed under natural circumstances (i.e. voluntarily, as would be done to humans). In animals that are repeatedly fed by gavage, it is likely that with time all animals would receive some antigen via the non-gastrointestinal route, and therefore might be expected to behave in a similar fashion. Voluntary feeding, such as was used in a recent oral GAD67 trial in NOD mice (93), is clearly the method of choice for studies involving 'oral tolerance', if these are to be clearly discriminated from trials which deliver the antigen by inhalation.

Mouse Strain and sex	Number of mice fed and analyzed	Number (%) of animals with +signal in plasma
NOD/Ltj female	23	10 (43)
NOD/Ltj male	10	4 (40)
NOR/Ltj female	12	5 (42)
NOR/Ltj male	4	1 (25)
BALB/c female	12	4 (33)
C57BL/6 female	10	3 (10)
Total	**71**	**27 (38)**

Table 2.1. Summary of results obtained for all animals fed radiolabeled bovine ß2-microglobulin by gavage and analyzed directly for protein in plasma.

Gavage Feeding	Voluntary Feeding
ability of animal to swallow impaired	animal able to swallow normally
knowledge of anatomical features essential	knowledge of anatomical features not necessary
damage to esophagus possible	no damage to esophagus
regurgitation/aspiration possible	voluntary nature makes regurgitation unlikely
forced nature of feeding makes overfilling of stomach possible	overfilling not possible
highly stressful, could potentially affect immune responses	minimal stress to animal

Table 2.2. Comparison of gavage and voluntary feeding methods in mice.

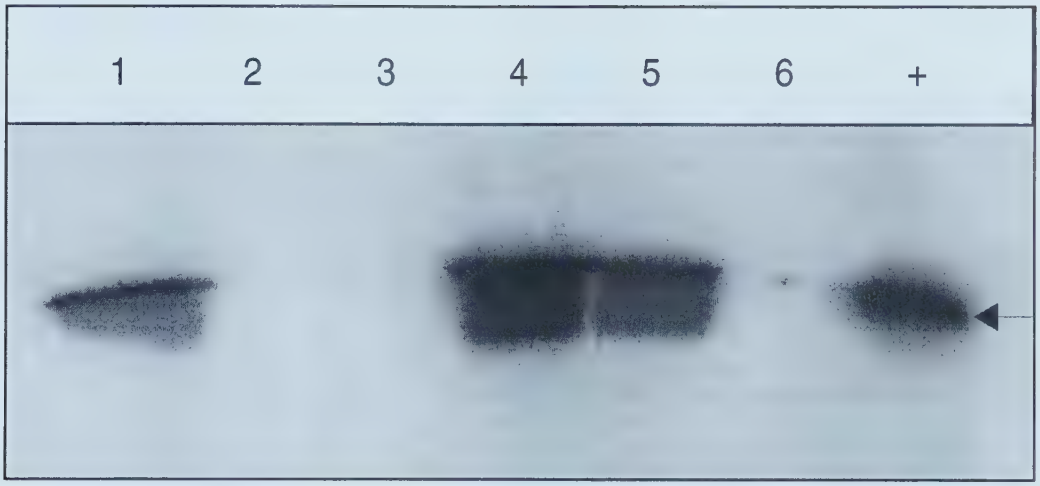


Figure 2.1. Autoradiograph showing SDS-PAGE of plasma samples (1µL) obtained 4 hours after mice were fed $1x10^7$ cpm of radiolabeled Bovß2-M via gavage. Unsedated BALB/c (lanes 1-3), sedated BALB/c (lanes 4-6). Positive control (+) is ^{125}I labeled Bovß2-M added to BALB/c plasma from control mouse. Arrow denotes bands corresponding to Bovß2-M.

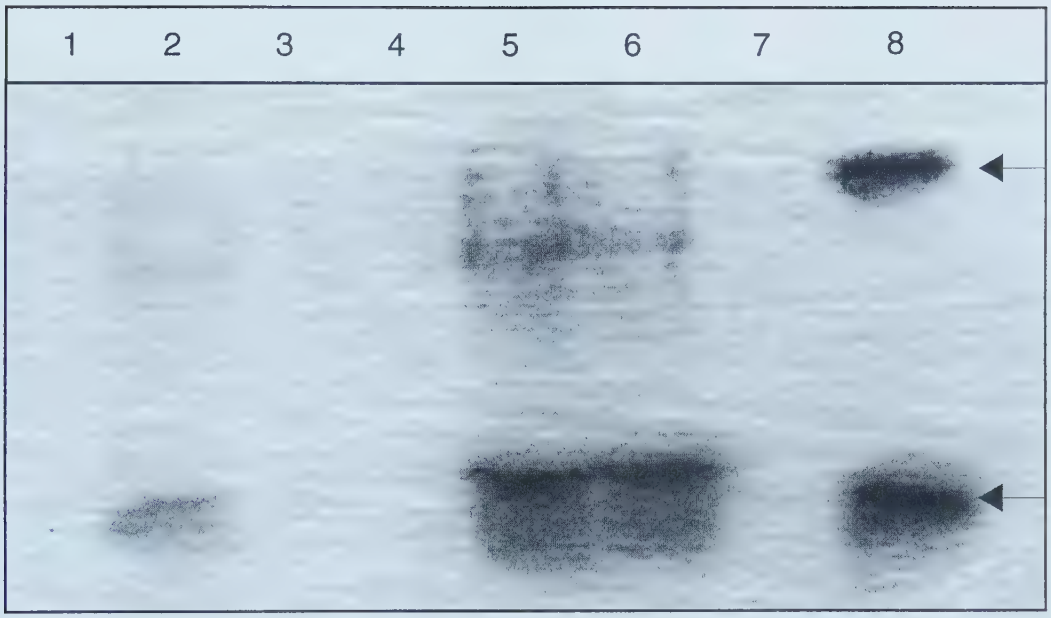


Figure 2.2. Sporadic transfer of Bovß2-M and BSA fragments into the plasma of mice. Autoradiograph of SDS-PAGE of plasma samples (1µL) from mice recieving equal counts of radiolabeled Bovß2-M ($1x10^7$cpm) and BSA ($1x10^7$cpm) via gavage 4 hours prior to bleeding. Female BALB/c (lanes 1-3), female C57BL/6 (lanes 4-6), negative plasma (lane 7) and control plasma with aliquots of the two radiolabeled proteins added (lane 8). Top and bottom arrow denote BSA and Bovß2-M standards respectively.

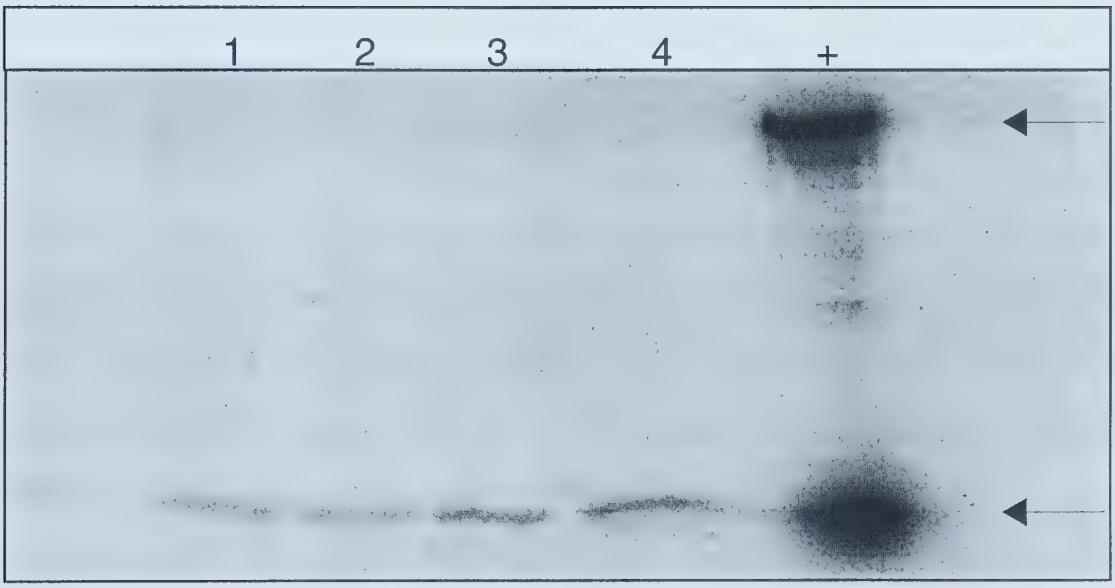


Figure 2.3 Autoradiograph from SDS-PAGE of immunoprecipitations of plasma from BALB/c mice which voluntarily drank a mixture containing equal counts ($1x10^7$cpm) of ^{125} I labeled Bovß2-M and BSA 4 hours prior to bleeding. Immunoprecipitations were performed on separate 100 µL samples of plasma using anti-Bovß2-M (CAB-297) or anti-BSA (BSA-33) monoclonals. Lanes 1 through 4 represent immunoprecipitations from 4 separate female BALB/c mice, with material from the Bovß2-M and BSA immunoprecipitations being pooled in a single lane for each animal. Upper and lower arrows denote BSA / Bovß2-M standards respectively, which are loaded in the (+) lane.

CHAPTER 3

ORALLY INGESTED BOVINE ß2-MICROGLOBULIN CAN PASS INTACT INTO THE CIRCULATION OF MICE AND PAIR WITH ENDOGENOUS CLASS I MHC: A POTENTIAL ADJUVANT MECHANISM FOR THE ASSOCIATION OF COWS' MILK CONSUMPTION WITH AUTOIMMUNE DIABETES

A. Abstract

Several studies have shown that levels of cows' milk consumption are positively correlated with incidence of IDDM. In an attempt to explain this correlation we considered the following hypothesis. Small quantities of ingested bovine beta 2-microglobulin (Bovß2-M), an immune globulin which is abundant in cows' milk, might enter the bloodstream intact and interact with immune-regulatory proteins (e.g. class I MHC heavy chains), thereby modulating the host immune response to a variety of environmental and/or self antigens and initiating the onset of IDDM. Using immunoprecipitation assays, we found that orally ingested radiolabeled Bovß2-M, fed by a voluntary drinking method, can pass intact and in a biologically active form into the small intestine and the circulation of adult NOD mice. There it can associate with class I MHC heavy chains on splenocytes, as well as with other class I-like proteins such as CD1 and FcRgII/III present on cells within the intestinal wall. In addition we found that Bovß2-M in circulation was deposited heavily onto vascular endothelium, and to a considerable extent within pancreatic islet cells. *In vitro* experiments indicate that Bovß2-M associates with the homologous human class I MHC, FcRgII/III and CD1d molecules lending supportive evidence for our novel hypothesis concerning the possible role of Bovß2-M as a dietary trigger for IDDM onset in humans.

B. Introduction

In 1984 Borch-Johnsen suggested that there was an inverse correlation between incidence of IDDM and duration of breast feeding (80). Further retrospective studies of IDDM patients quickly refuted this observation (81). Now more than ten years later, the controversy over the correlation between breast feeding and early cow's milk consumption with the incidence of IDDM continues. Several recent studies have found a strong correlation with cows milk consumption but not with duration of breast feeding (82,83). Based on these correlations, it has been proposed that cows milk may contain a triggering agent or co-factor for the development of IDDM although the nature of this agent remains undetermined. In previous work we were unable to demonstrate that cows' milk antigens are specifically cross-reactive with islet antigens. This led us to consider the following alternate hypothesis: that small quantities of bovine immune system proteins such as Bovine ß2-microglobulin (Bovß2M), which is present in cows' milk in concentrations up to 30 µg/ml, might be entering the bloodstream intact via interactions with molecules on the cell surface of the Intestinal epithelia and the M cells. Upon entrance into the circulation, Bovß2-M might be able to interact with mouse or human immune system proteins, thereby modulating the host immune response to a variety of environmental and/or self antigens.

ß2-microglobulin (ß2-M) is a single chain nonglycosylated polypeptide (approx. 11.5 kDa) which is found free in all body fluids of vertebrates (average concentration ≈1µg/ml), as well as in stable non-covalent association with MHC class I and class I-like heavy chains (1). ß2-M is the smallest member of the immunoglobulin gene superfamily, consisting of a single disulfide-linked immunoglobulin domain which has sequence homology (21-27%) to constant region domains of antibody heavy chains (1,22,23). ß2-M is absolutely required for the intracellular processing and transport to the cell surface of nearly all MHC class I heavy chains (20,21). However, once on the cell surface class I associated ß2-M is known to exchange with free ß2-M present in the plasma (*in vivo*) (22) or in tissue culture medium (*in vitro*) (23).

Both human and bovine ß2-M are known to efficiently exchange with mouse ß2-M on mouse class I MHC heavy chains resulting in xenogeneic heterodimers. *In vivo* studies with ß2-M knockout mice have shown differences in relative binding of exogenous ß2-M between different murine class I molecules (26). The difference in affinity of ß2-M for various different mouse heavy chains is a result of variations in amino acids between different class I haplotypes. These studies have indicated that in the mouse, heavy chains have the relative affinities for endogenous murine ß2-M in the order $K^b > D^d > D^b > L^d > K^d$. Conversely, a significant population of the mouse ß2-M "low affinity" heavy chains have been shown *in vitro* to be highly associated with xenogeneic, exogenous Bovß2-M from the fetal calf serum used in cell culture media (27-29).

The association of a xenogeneic ß2-M with a mouse class I heavy chain results in unique conformational epitopes which are recognized by CTL clones and complement fixing antibodies.(23,30) Some of the class I epitopes recognized after xenogeneic ß2-M ssociation are apparently located on the peptide binding, alpha 2 region of the heavy chain (31). Alterations in this region could affect the ability of the MHC class I / peptide complex to be recognized by T cell receptors of various subsets and alter the ability of class I molecules to bind exogenous peptides (e.g. dietary), thus indirectly affecting the ability of T cells to respond. Therefore, ß2-M not only plays a necessary role in class I cell surface expression and shaping the structure class I heterodimer, but also determines to some extent its overall function.

The interaction of endogenous ß2-M with other molecules is not limited to the classical class I molecules. It also pairs specifically with cell surface molecules such as the thymus leukemia antigen (TL), CD1 heavy chains, low affinity macrophage Fc receptors and the FcRn receptor. Some of these class I-like (class Ib) proteins are expressed at high levels in the intestinal epithelia throughout life, such as the TL antigen in mice and the CD1d antigen in humans (58,59). The high level of expression of these proteins in this area suggests that exogenous ß2-M in the small intestine would be able to interact with these and

possibly other proteins. In adults, some portion of orally ingested bovine immunoglobulins have been shown to be able to pass through the stomach and small bowel without being digested (94). The interaction of these proteins with molecules on the cell surface of the Intestinal epithelia and the M cells have been implicated as a causative agent of conditions such as severe chronic infantile colic (95). As ß2-M has significant sequence identity (21-27%) to immunoglobulin heavy chain constant regions (2) it seemed likely that it would be found intact and perhaps biologically active (i.e in a native conformation) in the small intestine.

As we had previously determined that there was high levels of biologically active Bovß2M in commercial milk we began a series of *in vivo* experiments where nonobese diabetic (NOD) and other strains of mice were voluntarily fed highly radioactively labeled purified Bovß2-M. After ingestion of the labeled protein, various organs and total plasma were analyzed for the presence of free or cell surface associated Bovß2-M between the two strains.

C. Materials and methods

Mice

BALB/c and C57BL/6 mice (female, 8 week), as well as NOD/Ltj and NOR/Ltj mice (male or female, 8-24 week) were obtained from local breeding colonies maintained by the University of Alberta Health Sciences Laboratory Animal Services (HSLAS), or from Jackson Laboratories (Bar Harbor, ME). All procedures involving animals received the approval of the local Health Sciences Animal Welfare Committee, and experiments were carried out under consultation with the veterinary and technical staff of the HSLAS.

Cell lines

Homozygous EBV transformed human typing cells WT-51 (GM3103B), WT-49 (GM3098A) and AMA-I (NIGMS Human Genetic Mutant Cell Repository at the Coriell Cell Repository) were grown in RPMI with L-glutamine+10% human serum without antibiotics. The mouse thymoma cell line A20-11.1 (D^d,K^d) was grown in PFHM-II

(Gibco BRL, Life Technologies Inc., Rockville, MD) and the mouse macrophage like cell line PD3881 (Coriell Cell Repository) was grown in RPMI with 5% fetal calf serum.

Proteins and radiolabeling

Bovine serum albumin (Fraction V, RIA grade) was purchased from Sigma Chemical Co. (St. Louis. MO), and Bovß2-M was isolated from bovine colostrum via the method of Groves and Greenberg (86). Proteins were labeled by a tyrosine oxidative iodination. Bovß2-M (500µg) or BSA (500µg) in 500µL of PBS pH 5.6 was added to glass tube (12mm x75mm Fisher Scientific, Mississaugua, ONT.) with one IODO-BEAD® per tube (Pierce, Rockford, IL). 5mCi $Na^{125}I$ (IMS.300 Amersham, Arlington Heights, IL) in a vol of 9µL was then added to the solution. The iodination reaction was allowed to proceed at 20°C for 7 min, and was terminated by simply removing the liquid from the glass tube and the Iodobead with a pasteur pipette. To separate free ^{125}I from labeled protein the reaction mixture was passed through a size exclusion column consisting of a 10 ml polypropylene syringe with 5 ml (bed volume) sephadex G-10 (Pharmacia, Sweden) equilibrated in PBS pH 5.6. Fractions (0.5 ml) were collected and 1µL of each fraction was transfered to the appropriate tube and counted with a LKB Wallac 1275 gamma counter. Peak fractions were combined and TCA precipitable counts determined as follows: 0.5 ml of 1% BSA (w/v) in PBS pH 5.6 was added to four glass tubes (12mm x75mm Fisher Scientific, Mississaugua, ONT.) each containing a 25 µl aliquot of the pooled column fractions. One-half of a ml of PBS pH 5.6 was added to two tubes, and 0.5 ml of 10% TCA in PBS pH 5.6 was added to the other two tubes. The tubes were placed on ice for 15' then centrifuged at 500 x G at RT for 15'. Twenty µl of supernatant was removed from each tube and counted. The percentage of protein associated counts (PAC) were determined by the following calculation;

$$\%PAC = \frac{\text{(supernatant from pooled aliquot + PBS) - (supernatant from pooled aliquot + PBS/TCA)}}{\text{(supernatant from pooled aliquot + PBS)}} X100\%$$

Protein associated counts ranged from 95-98%, and specific activity was determined by measuring the absorbance of labelled solution at 280nm. The specific activity of Bovß2-M was ≈ $1x10^6$cpm/µg and BSA specific activity was ≈ $2x10^6$cpm/µg.

Feeding

The voluntary feeding method is described in detail in chapter 2. In brief, mice were trained to drink a 1% sucrose / PBS pH 5.6 mixture from a 1 ml syringe by repeatedly removing the regular water bottle overnight and then holding the syringe out for them to drink from in the morning. Before feeding labelled protein, mice were isolated in metabolic cage without food or water for 12 hours to allow for clearance of the food bolus from the stomach and early small intestine. Mice were then allowed to drink a mixture of ^{125}I labeled Bovß2-M ($1x10^7$cpm) and ^{125}I BSA ($1x10^7$cpm) from a 1 ml syringe. Mice having ingested labelled protein were left in metabolic cage for a further hour without food or water, following which they were both returned to allow as stress free an environment as possible.

Injections, histology, and in situ autoradiography

Mice received injections of ^{125}I-radiolabeled Bovß2-M (≈7 µg of Bovß2-M in 100 µl of phosphate buffered saline pH 5.6, specific activity ≈1 x 10^6 cpm/µg) via the penile vein, and 4 hours later were anesthetized using Metophane™ (Janssen Pharmaceutical, New York, ONT) and sacrificed by cervical dislocation. Control mice injected with PBS only were harvested at the same time. The spleen, pancreas, liver, and right kidney were harvested and fixed in 10% buffered formalin (3 changes over 3 days at RT). Tissues were dehydrated by passage through increasing concentrations of ethanol (70-100%), placed in three changes of 100% xylene over 2 h and then embedded in paraffin. Five micron sections were cut from tissue blocks using a microtome, floated on a warm water bath, and mounted on glass microscope slides. Slides were de-waxed in xylene, dried, and then dipped in liquid photographic emulsion (Eastman Kodak, Rochester, New York) and stored in the dark. After 2 weeks or 6 weeks slides were developed using standard

photographic developing solutions, and before the plastic emulsion was completely dry, most slides were lightly stained with hematoxylin and eosin (H and E) to visualize the tissue architecture. Slides were viewed and photomicrographs taken on a standard light microscope. Control sections showed less than three silver grains per high-powered field.

Collection of Blood

Six hours after feeding labeled protein mice were anaesthetized with Metophane™ and bled to termination via cardiac puncture. Whole blood (typically 500µl volume) was collected into eppendorf microfuge tubes already containing 100µl sodium heparin (10% v:v). Plasma was isolated by spinning the unclotted blood at 500 x G at 4°C for 10 min. Plasma was removed from cells (≈ 250uL), and placed in a fresh 1.5 mL eppendorf microfuge tube, at this point the plasma was either immunoprecipitated immeadiately or stored at -20°C. Pellet of cells was stored at -20°C for future use if needed.

Isolation of Cells from spleen and small intestine

Spleens were removed from exsanguinated mice and a single cell suspension prepared by homogenizing the tissue between two frosted microscope slides and washing it into 10 ml RPMI. Splenocytes were then purified on Lympholyte®-M (Cedar Lane, Hornby , ONT) as per manufacturers instructions. Briefly, for each mouse spleen 3 X 3ml of Lympholyte®-M (prewarmed to RT) were placed into three 15 ml conical tubes, and gently overlayed with splenocyte suspension (2.5ml/tube). The remaining homogenized tissue RPMI suspension was discarded. This was centrifuged at 1000 X G at RT for 20' , and splenocytes recovered from the interface using a pasteur pipet.

When comparing the differential binding of Bovß2-M to the class I molecules (D^b, K^d) in NOD and NOR mice, splenocytes were prepared as above, but were counted using a hemocytometer. Equal numbers of splenocytes ($8x10^7$) were then lysed and immunoprecipitations performed.

Following cardiac bleed, intestinal wall cell suspensions were obtained by removing the entire small bowel, which was opened longitudinally and washed x3 in

PFHM-II (25 ml). The tissue was then cut into 0.5 cm lengths and macerated in 10 ml PFHM-II between two frosted microscope slides. Larger tissue fragments (>3mm diameter) in the resulting mixture were allowed to settle for 5 min and the top fraction (≈5 ml) was removed and layered on top of Lympholyte M (10 ml) in a 15 ml Falcon tube. Tubes were spun at 1000 x G for 20 min at 20°C and the upper layer and interface collected, transferred to a 15 ml falcon tube already containg an equal vol of PFHM-II, the cells were then pelleted at 500 x G for 5' at 4°C. The cell pellet was washed once with 10 ml PFHM-II prior to lysis and immunoprecipitation.

Immunoprecipitations

Single cell suspensions of cell cultures or Lympholyte®-M (Cedar Lane, Hornby, ONT) purified splenocytes or intestinal wall cells were lysed with 2 ml of lysis buffer (1% NP40, 150 mM NaCl, 20 mM TrisHCl pH7.5) per $1x10^7$ cells. The lysate was incubated with agitation at room temperature for 5 min and then spun at 1200 x G at 4°C for 5 min to remove debris. The supernatant was pre-cleared with 2µg of relevant isotype matched control (L243 for mouse and B22-249 for human) on rotor at 4°c for 12 hours to keep beads suspended. 300 µl of a slurry of cross linked protein A 4B-CL sepharose/PBS (50 mg/ml dry weight) was added and the tubes rotated for an additional 12 h at 4°C. Supernatants were removed and divided into 2 aliquots and placed into fresh tubes. 2µg of the McAb of interest were added to one aliquot with 2µg of isotype matched control in the other aliquot. procedure above for pre-clear was repeated. McAb's and specificities were as follows; SF1.1.1 (Kd), B22-249 (Db), 1H1 (mouse CD1 and human CD1d) (Pharmigen #09861D (San Diego, CA)), anti FcR(CD16/CD32) (Pharmigen # 01241A), L243 (HLA-DR), CAB-297 (anti Bovß2-M), BSA-33 (anti BSA) (Sigma Chemical Co., St. Louis, MO). After incubation supernatants were removed and frozen. Sepharose beads were washed twice with 1 ml of wash buffer (0.2% NP40, 150 mM NaCl, 10 mM TrisHCl pH 6.8) and once with 1 ml of PBS pH 6.8. Excess supernatant was removed and SDS loading buffer added. The samples were heated at 85°c for 5' and loaded immediately.

Plasma immunoprecipitations (≈100 µl plasma) were performed as above with the pre-clear consisting of 500 µl of the protein A/PBS slurry and no isotype matched control McAb. A larger amount of protein A sepharose was used to bind the mouse IgG present in plasma. Plasma was separated into 2 equal aliquots, 2µg of McAb CAB-297 was added to one aliquot and to the other was added 2µg BSA-33 (Sigma Chemical Co.). Before the addition of SDS loading buffer to the washed beads, precipitated BSA and Bovß2-M beads were combined.

Electrophoresis and imaging

All SDS-Page was performed on a BioRad® MiniGel™ apparatus using a 5% acrylamide stacking gel and a 15% separating gel. Gels were run at a constant 25mA/gel for 50 min. Gels were fixed and stained in a 0.5% comassie blue G250 50% MeOH / 10% acetic acid solution for 15 min and destained in 10% MeOH / 10% acetic acid solution for 1 h. All gels were vacuum dried at 80°C and placed on a phosphorimager plate (BAS-IIIs, Fuji, Japan) for 3 days.

D. Results

Binding of Bovß2-M to Intestinal class I like Molecules *in vivo*.

The binding of exogenous Bovß2-M has previously been reported for a series of class I and class I like molecules in vitro. However, heterodimerization with Bovß2-M after ingestion has not been reported. When we immunoprecipitated various class Ia/b proteins from cells within the intestinal wall, we found ^{125}I labeled Bovß2-M to be non-covalently associated (figure 3.1). This indicates that Bovß2-M is able to pass through the harsh conditions of the stomach and reach the intestine intact. Furthermore, the protein retains biological activity after passage based on its ability to bind to class I molecules, CD1d and to a lesser extent FcRgII/III from intestinal cell lysates (figure 3.1). Interestingly, in other experiments (data not shown) we examined neonatal mice whose mothers had been fed

labeled Bovß2-M; here we found the presence of an altered, possibly dimerized Bovß2-M in the stomachs of the suckling mice.

Preferential transport of Bovß2-M into circulation

We found there is preferential transport of Bovß2-M, compared to BSA, into circulation from the intestine in a variety of strains of female mice. Autoradiographs of plasma immunoprecipitations performed with anti Bovß2-M and BSA show detectable amounts of full sized Bovß2-M in murine circulation after voluntary ingestion (figure 3.2). The absence of the BSA control band indicates that this is a protein specific effect and not the simple passage of protein in general through lesions in the gastrointestinal tract or via regurgitation into the lungs. After passage into the circulation, orally ingested ^{125}I-labeled Bovß2-M was able to associate with D^b molecules on NOD and NOR splenocytes (figure 3.3), indicating that biological activity of this molecule is not affected by transport across the intestinal wall. In comparison to D^b, relatively minor amounts of Bovß2-M was found associated with the K^d molecule in NOD mice, whereas no Bovß2-M was found associated with immunoprecipitated K^d in lysates made from equal numbers of NOR splenocytes (figure 3.3).

Tissue deposition of Bovß2-M

We found that Bovß2-M was preferentially deposited onto endothelial cells, red blood cells, splenocytes, and pancreatic islet cells after IV injection. It was also deposited preferrentially in inflamed tissue such as NOD islets undergoing lymphocyte infiltration (figure 3.4).

Binding of unequal amounts of Bovß2-M to different human class I *in vitro*

While binding of Bovß2-M to human class I is known to occur, we were interested to see if there was any appreciable difference in the binding affinity of various human class

I haplotypes to Bovß2-M. Incubation of human EBV typing cells, WT51, AMA-1 and WT49 with ^{125}-I labeled Bovß2-M followed by immunoprecipitations with McAb W6-32 (specific for all classes of human class I) showed that exchange of Bovß2-M is possible on a variety of different human class I molecules (figure 3.5). Significant differences in Bovß2-M exchange was observed for different haplotypes, with the typing cell line AMA-1 binding four times more Bovß2-M than the WT49 typing cell (figure 3.6B). To determine if this was the result of a difference in the amount of class I on the cell surface between the two lines, class I staining of the cells was performed in parallel. In comparing cell surface class I density by flow cytometry, virtually no difference was found in the levels of class I between the two cell lines (figure 3.6A).

E. Discussion

Intact transmission and intestinal binding

We found that Bovß2-M can be orally ingested by various strains of mice and pass intact and biologically active into the small intestine. Once in the small intestine Bovß2-M can associate with various molecules on cells of the intestinal wall including class I, CD1 and to a lesser extent FcRgII/III. We have also found that 6 hours after ingestion of Bovß2-M, detectable levels of intact protein can be found in the plasma of these mice and also associated with class I on splenocytes. The transport of intact dietary maternal antibodies into fetal and neonatal circulation by FcRn receptors during early stages of developement has been well documented (46). The cessation of this passive immune transfer occurs at defined times post conception for various species (48). To our knowledge, Bovß2-M transfer is the first example of transmission of intact dietary protein into the circulation of an healthy adult animal.

Bovß2-M interactions with class I, CD1 and FcRgII/III on the small intestine luminal surface may be responsible for the specific transport of Bovß2-M into the circulation of mice. However, it seems likely that additional proteins or non-specific

interactions are involved. In experiments where transport through the stomach was efficient (i.e. via gavage feeding) a significantly larger proportion (≈30%) of ^{125}I labeled protein bound strongly to the lumenal surface of the small intestine (data not shown) compared to the relatively smaller amounts found by immunoprecipitation of class I, CD1 and FcRgII/III from cells extracted from the wall of the small intestine. Thus, while not accounting for the major proportion of intestinal wall associated Bovß2-M, these molecules remain attrative candidates due to their localization to and rescue from endocytic vesicles in the intestine followed by their transport back to the cell surface (48). This recycling process provides an intriguing model for the uptake and internalization of associated exogenous Bovß2-M. In addition the FcRn receptor seems a likely candidate for our putative Bovß2-M transporter (especially in the neonatal period) as it is highly expressed on the intestinal wall during early development and has a undisputed transport function there.

Like class I the FcRn heavy chain requires ß2-M association for function. However it was not known in what manner, exogenous Bovß2-M might interact with this receptor in the neonate. Our results showing Bovß2-M association with the structurally similar FcRgII/III receptors implies that a similar association could occur with the related FcRn receptor. Curiously the outer membrane layer of the placenta called the syncytiotrophoblast expresses functional FcRn but is not believed to express ß2-M. If this is true it is not clear how the FcRn could function in the transport of maternal antibodies from mother to the fetal circulation here. It is conceivable that the FcRn heavy chain expressed on the syncytiotrophoblast could bind ß2-M (or Bovß2-M) from exogenous sources such as free ß2-M in maternal plasma thus allowing it to form a functional heterodimer. This would be an elegant example of the symbiotic realationship involved in the developement of an infants immune system while inside its mother.

Structural studies of the FcRn/ß2-M/ IgG trimer have shown it to be very stable at the acidic pH found in the placental endocytic vessicles and in the intestinal lumen of mice(50). Once the trimer reaches the more neutral pH of the vasculature it loses its stability

and releases the bound IgG into the circulation. This destabilization could also result in the loss of weakly associated Bovß2-M, leaving the unfolded FcRn heavy chain to be recycled back to the cell surface. Once there the FcRn heavy chain could associate with additional exogenous ß2-M or Bovß2-M and with IgG bound be transported across the syncytiotrophoblast into the fetus creating a recycling "pump" for moving Bovß2-M (as well as maternal antibody) into the fetal circulation.

The murine class Ib molecule CD1 (homologue CD1d in humans) is another attractive candidate for a potential Bovß2-M transport molecule. It is highly expressed by the intestinal epithelia (60) and plays a role in presenting a limited repertoire of peptides to a subset of intestinal epithelial lymphocytes (IELs) (61). It appears that the human CD1d molecule is unique in its ability to reach the cell surface without ß2-M associated, as all other members of the human CD1 family require ß2-M to be expresssed in order for the larger α chain to reach the cell surface (55). CD1d is structurally distinct from the other members of the CD1 family and this difference may play a role in its atypical expression (60). A non glycosylated, ß2-M free CD1d molecule is believed to be a major constituent present on the luminal surface of human intestinal epithelial cells (56). If this is true CD1d would provide an obvious intestinal Bovß2-M ligand/transport molecule as it requires exogenous ß2-M for function. Intestinal class I-like presentation dependant on exogenous ß2-M would presumably involve exogenous dietary peptides, a feature of the Cd1 molecules which has previously been postulated . Reports of ß2-M dependency of the murine CD1 protein cell surface expression have been made and while it is certain that this is true in developing thymocytes discordant results in FO - transfectants have been reported, leaving the peculiarity of human CD1d expression and the discrepancy between it and its murine homologue unresolved.

Recently discovered functions of the CD1d molecule offer exciting explanations for our postulated Bovß2-M /IDDM correlation. Most recently, studies have implicated the CD1 family as antigen presenting molecules for a highly specialized, IL4/IFN-gamma

secreting T cell subset (Wilson and Hafler, in press in Nature). It appears that the role of this T cell subset may be in determining whether a TH1/TH2 biased response predominates by introducing high levels of cytokines at an early stage in the immune response. The CD1 molecule presents antigens to a small population of T cells that express the NK1 cell surface C-type lectin. This subset of T cells are phenotypically CD4+/CD8- or CD4-/CD8- but they share the peculiar feature of an invariant TCR- alpha chain (Va24JaQ+) which pairs preferentially with a limited set of ß chains (Vß8, 7 or 2). These cells also share the ability to secrete large amounts of IL4 after stimulation with anti CD3 *in vitro.*

Interestingly, the analogous murine lymphocyte population in two animal models for autoimmune disease, the mouse strains SJL and NOD/Ltj, was reduced 3-5 fold and was severely defective in it's ability to secrete IL-4. Recently Wilson et al. showed in a pair of identical twins discordant for Type I diabetes, the diabetic sibling had lower numbers of the CD4-/CD8- Va24JaQ+ T cells than the unaffected sibling. In contrast Va24JaQ+ T cell clones isolated from the diabetic sibling were only capable of secreting IFN-gamma while clones from their unaffected siblings were able to secret both IL-4 and IFN- gamma (Wilson and Hafler, in press in Nature). The effect of Bovß2-M associated CD1 molecule on stimulation of these lymphocytes is unknown but could potentially affect the limited repertoire of peptides that CD1 is able to bind as in class I and thus change the peptides that stimulate these specialized cells.

The ability of Bovß2-M to associate with CD1 could explain some of the perplexing features of the immunological nature of the small intestine. Intra-epithelial lymphocytes (IELs) are known to possess different phenotypic populations than those found in the peripheral blood and other immune related organs. Of major interest is the greatly increased proportion of gamma delta T cells that are CD8+ in the IELs. Surprisingly, this subset appears to be unaffected in ß2-M knock out mice, giving rise to speculations concerning radically different forms of antigen sampling and class I like restriction occuring there. This observation presents a conundrum to traditional T cell developement models, in which

functional class I is required for the positive selection of a CD8 + cell. Suggestions that these cells are in some way restricted to a class I like /CD8 ligand such as the CD1 or TL have arisen. Of these candidates the CD1 molecule (mouse homologue of human CD1d) is extremely attractive due to its ß2-M independent expression. However it is unlikely that it can present antigen to T cells without associated ß2-M which leaves the presence of CD8+ cells in ß2-M knockout mice unresolved (personal communication, Steven Porcelli). Based on our results it is possible that a supply of ß2-M is originating from their diet of mouse chow , in which protein is typically bovine based, allowing presentation of peptides by the CD1 molecule and thus allowing education and positive selection to occur in the intestine.

Plasma and Cellular Interactions

In our studies, outside of the small intestine detectable levels of Bovß2-M could be found only free in circulation or associated with class I MHC heavy chains. It is not surprising that no FcRgII/III or Cd1 associated Bovß2-M was found in splenocyte lysates due to the extremely low level of intact protein present in the circulation. In addition our *in vitro* results indicated that no other class I like molecule studied bound Bovß2-M to the same degree as the classic class I proteins. Our studies also indicated that as expected the D^b class I molecule bound the majority of class I associated Bovß2-M compared to the K^d molecule. The NOD mice studied had small amounts of K^d associated Bovß2-M while the NOR mice had none. As there was no difference observed in absolute amount of Bovß2-M transferred into circulation between NOD and NOR mice, the differential binding of the identical K^d molecule is presumably a result of the relative affinity of K^d for the different alleles of murine ß2-M that are present in these mice. Where the "A" allele in NOD mice is more easily replaced by Bovß2-M than the "B" allele in NOR. Interestingly the only murine T cell clones that have been isolated against islet antigens are K^d restricted (110).

Deposition

The histology of NOD mouse tissue after ^{125}I Bovß2-M IV injection showed that there was high levels of deposition in endothelial cells, splenocytes and interestingly in the

pancreatic islet cells. The association of Bovß2-M to splenocyte cell surface proteins was expected from earlier immunoprecipitation experiments, however higher deposition on islets undergoing mononuclear cell infiltration was of great interest. Up regulation of cell surface class I by IFN gamma is a well documented phenomenon, presumably allowing even greater amounts of Bovß2-M to be bound by activated lymphocytes than by resting ones. We are currently evaluting the use of labelled Bovß2-M for imaging areas undergoing immune activation as it has great potential in clarifying this process in histological studies of tissue.

The preferential deposition of Bovß2-M onto endothelial cells may explain the low levels of free Bovß2-M found in the plasma of the mice studied. This deposition would be expected as animals recieving IV injections would initially have relatively high levels of Bovß2-M in the circulation. Bovß2-M exhibits a high degree of non specific interactions with itself and other proteins when present in concentrations that might be experienced post injection (data not shown). In addition to non specific interactions, the endothelium expresses class I and other class I-like proteins such as the FcRp receptor (responsible for protecting circulating IgG from catabolism) which is present in high levels on the endothelial cell surface and is identical to the FcRn receptor. This molecules requires an associated ß2-M to function and may also be involved in the high level of Bovß2-M deposition (51) as well as uncharacteristically long plasma half life observed for Bovß2-M in the mice studied (see Chapter 4).

It is of great interest that high levels of Bovß2-M are deposited in the islets. While any tissue that is glandular in nature would presumably have high levels of circulating Bovß2-M present, the inherent physical property of the islets ensures exposure to any circulating plasma peptides and proteins. The islet vascular endothelial cells possess specialized fenestrations which allow the passage of low molecular weight molecules, offering no restriction to ß2-M passage and would allow Bovß2-M free access to the ß cells. Once inside the islets, Bovß2-M could alter the structure of the class I heavy chains

and potentially initiate a cytolytic response towards the ß cells. A combination of fluctuating Bovß2-M levels along with the presence of dietary peptides would make the islets an ideal site where cross reactive antigens could be taken up by class I and initiate additional cytolytic responses, eventually triggering the immune systems progression towards IDDM.

The potential effects of having biologically active Bovß2-M interacting with cellular components through out the body is wide ranging. ß2-M has been shown to act as an adjuvant which specifically induces CTL responses (7), although the mechanism has not been determined. It is possible that fluctuations of Bovß2-M in the circulation, resulting from consumption of biologically active Bovß2-M in dairy products, could cause a high level of cytolytic stimulation. Experiments showing increased uptake of exogenous peptide in the presence of ß2-M suggest that *in vivo* mixtures of dairy protein peptides and Bovß2-M, resulting naturally from dairy consumption, would promote dietary peptide loading onto misfolded or conformationally incorrect class I. We have developed two possible models which explain the potential for Bovß2-M class I association to stimulate cytolytic responses (figure 3.7).

The first model involves the rescue of an unfolded, peptide free class I heavy chain by association of Bovß2-M. As the heterodimer forms, exogenous peptide is bound in the peptide binding groove. This process would effectively uncouple display of endogenous peptides on class I to tolerized T cells, and allow recognition, activation and subsequent cytolytic attack of essentially normal cells displaying peptides which originated outside of the cell. The second model describes a more allogeniec type response where the cooperative exchange of endogenous ß2-M with Bovß2-M results in altered conformation of the epitopes recognized by the T cell receptor as "self". This would stimulate a series of responses which could potentially cascade, affecting surrounding tissues much like a classic host versus graft response. Interestingly, histological studies of the cytolytic islet attack on islet cells in IDDM patients have been compared to that seen in graft rejection. As

the actual mechanism of ß2-M exchange on class I is still in contention, either of these models could be correct.

Bovß2-M's ability to cross into circulation necessitates the reinterpretation of *in vivo* studies where the presence of xenogeneic ß2-M was considered impossible. In one report immunoaffinity purification of human ß2-M from human urine was accomplished using an anti-ß2-M McAb: as well as purifying human ß2-M, a second protein was also co-purified which strangely had a pI identical to that of Bovß2-M (11). Another protein with the same pI was also found in similar *in vitro* studies which was identified as Bovß2-M. The possibility that the protein from human urine was Bovß2-M was discounted as neither the urine nor the monoclonal antibody had ever come in contact with bovine proteins. Based on our observations this assumption cannot be considered correct. It is conceivable that the concentrations of Bovß2-M in a patient suffering from renal failure would be detectable by an extremely sensitivite radioactive assay as was being used.

If the passage of Bovß2-M into the circulation of humans and mice has relevance to the development of IDDM, then the association of autoimmune diabetes with conditions where increased uptake of intact protein through the intestinal wall occurs would support a causal link. In this regard epidemiological studies have found a correlation between increased number and frequency of enteroviral infections and the onset of IDDM. Infections of this nature are known to cause inflammation of the small intestine and increased intestinal uptake of dietary proteins and peptides. The correlation of enteroviral infection with IDDM has traditionally been attributed to the presence of viral / islet cross-reactive antigens. We propose the alternate hypothesis that autoimmunity could result from the increased passage of Bovß2-M and/or other dietary peptides into the circulation and their deposition onto cells in a variety of tissues including the immunologically sensitive/vulnerable islets. Other epidemiological studies have drawn correlations between IDDM and dairy consumption only in early stages of life (e.g. first year), not during later stages. Intact protein uptake is known to occur at greater levels in children than in adults

and could result in increased levels of Bovß2-M exposure internally and the observed correlation.

Extending our work into human studies has been hampered by lack of reagents that would allow us to determine if similar transport of Bovß2-M is possible into the circulation. We are currently developing competitive radioactive immunoassays to detect this phenomena. At this point this assay has suffered from unexpected non-specific effects which suggest that there are other proteins present in sera that interfere with monoclonal antibodies binding to Bovß2-M. This may be a result of ß2-M plasma binding proteins that have been describe previously in healthy humans. Other methods of labeling Bovß2-M are being considered as alternative options for following the fate of this protein in humans, as ^{125}I-labeling is unacceptable in view of the deposition of labeled protein onto the intestinal epithelium observed in mice.

Further studies to determine if the levels of transport in mice and humans could be considered sufficient to contribute to immune disregulation are being considered. We propose that further studies of lymphocyte populations of ß2-M deficient mice be performed with mice receiving diets that have no possibility of containing Bovß2-M, as common mouse diets typically contain complex protein mixtures that originate from bovine sources and could affect the CD8+ cell developement and immune response in these animals. Use of ß2-M knock out mice in our studies will allow us to observe the effect of a constant dietary supply of exogenous xenogeneic ß2-M on the development or lack thereof of functional class I responses. Defined infant formulas which contain no complex proteins or peptides are now available. Recent studies using these formulas with NOD mice in pathogen free environments show that these mice do not develop diabetes. We are in the process of determining if the addition of purified Bovß2-M +/- a single purified protein (e.g. ovalbumin) to these peptide/protein-free diets will restore the autoimmune responses leading to diabetes which is characteristic of the NOD strain.

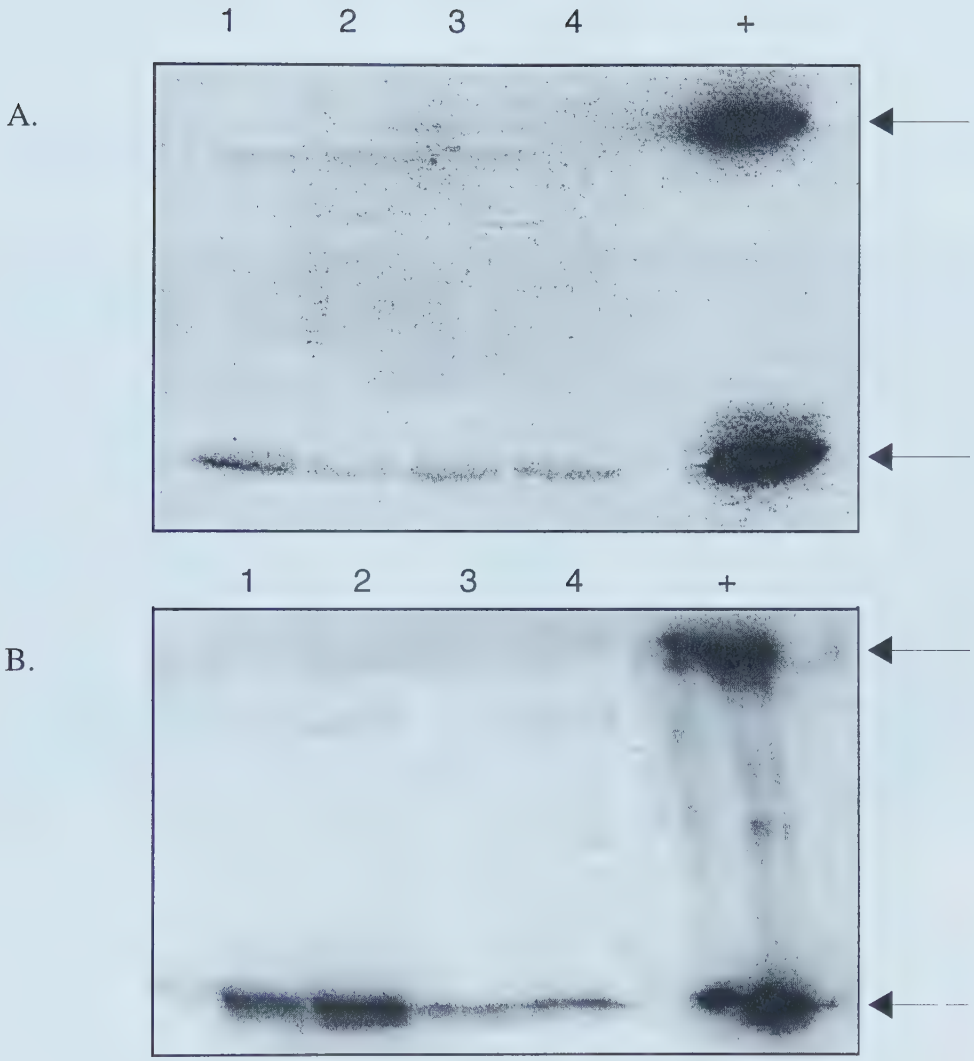


Figure 3.1. Autoradiographs from SDS-PAGE of immunoprecipitations of plasma from A) NOD/Ltj and B) C57BL/6 mice which voluntarily drank a mixture containing equal counts ($1x10^7$cpm) of ^{125}I labeled Bovß2-M and BSA 4 hours prior to sacrifice. Immunoprecipitations were performed on separate 100 µL samples of plasma using anti Bovß2-M (CAB-297) or anti-BSA (BSA-33) monoclonals. Lanes 1 through 4 represent immunoprecipitations from 4 separate female mice, with material from the Bovß2-M and BSA immunoprecipitations being pooled in a single lane for each animal. Upper and lower arrows denote BSA / Bovß2-M standards respectively, which are loaded in the (+) lane.

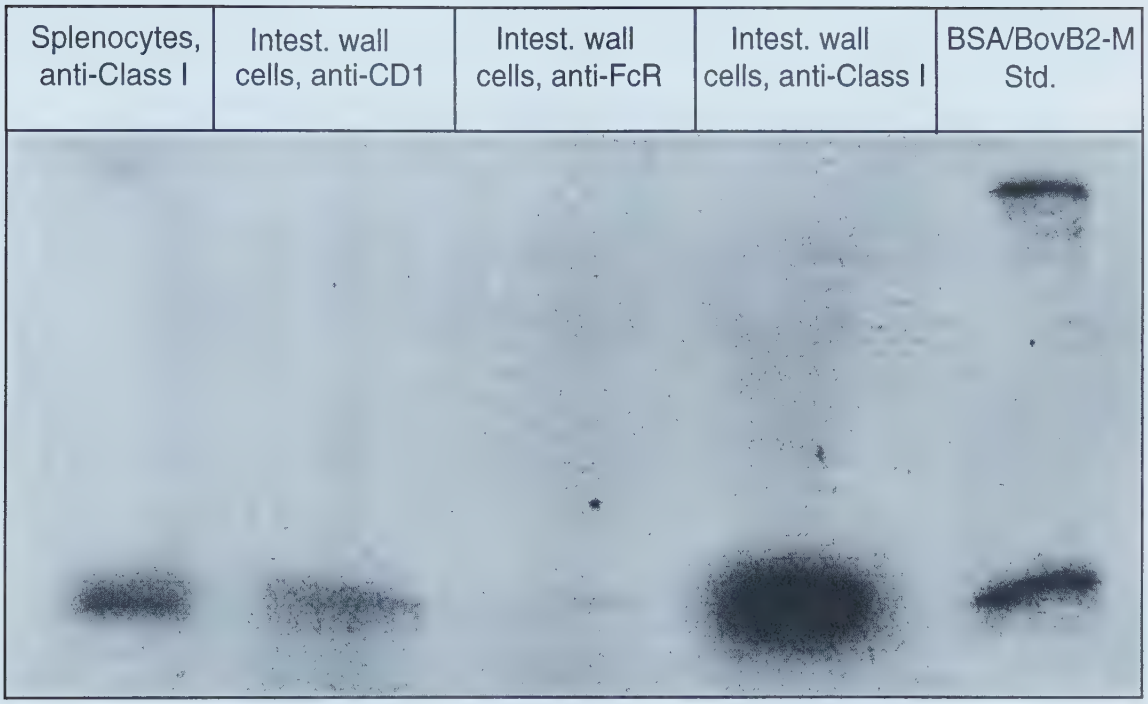


Figure 3.2. Autoradiograph of immunoprecipitations of various Class I and Class I-like molecules from a NOD/Ltj female mouse sacrificed 4 hours after voluntarily ingesting ^{125}I labeled Bovß2-M ($5x10^8$ cpm). Single cell suspensions derived from the small intestine wall and the spleen were both purified on Lympholyte-M gradients, and aliquots of the resulting cell lysates were immunoprecipitated seperately using monoclonal antibodies specific for class I (combined monoclonal against K^d (SF1.1.1) and D^b(B22-249)), for CD1 (1H1), or for FcRII/III (2.4G2).

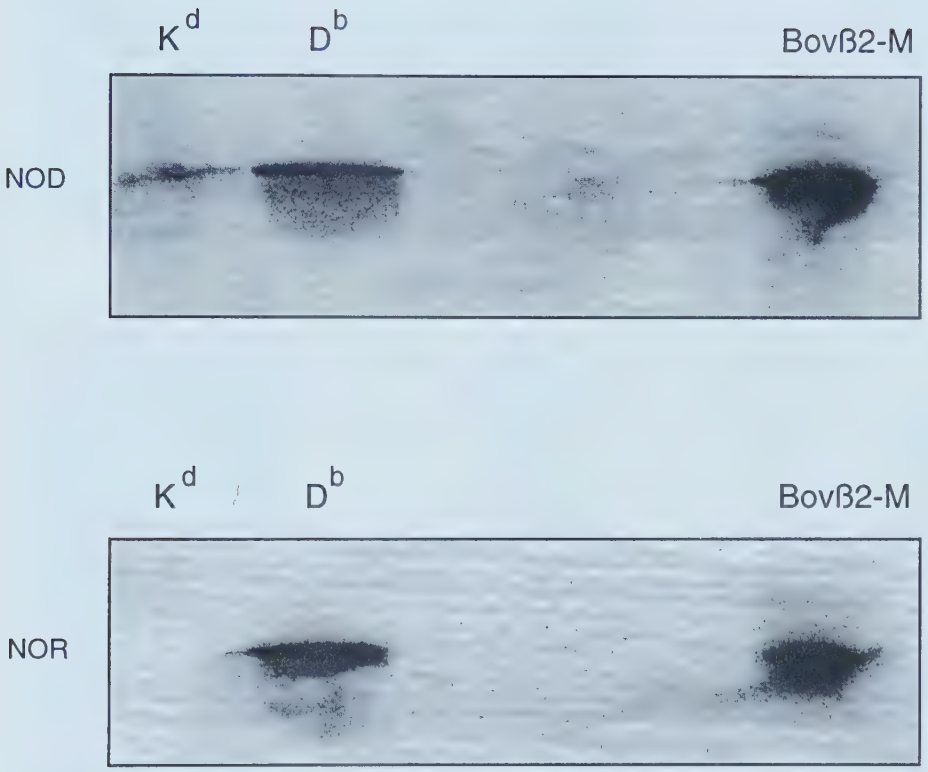
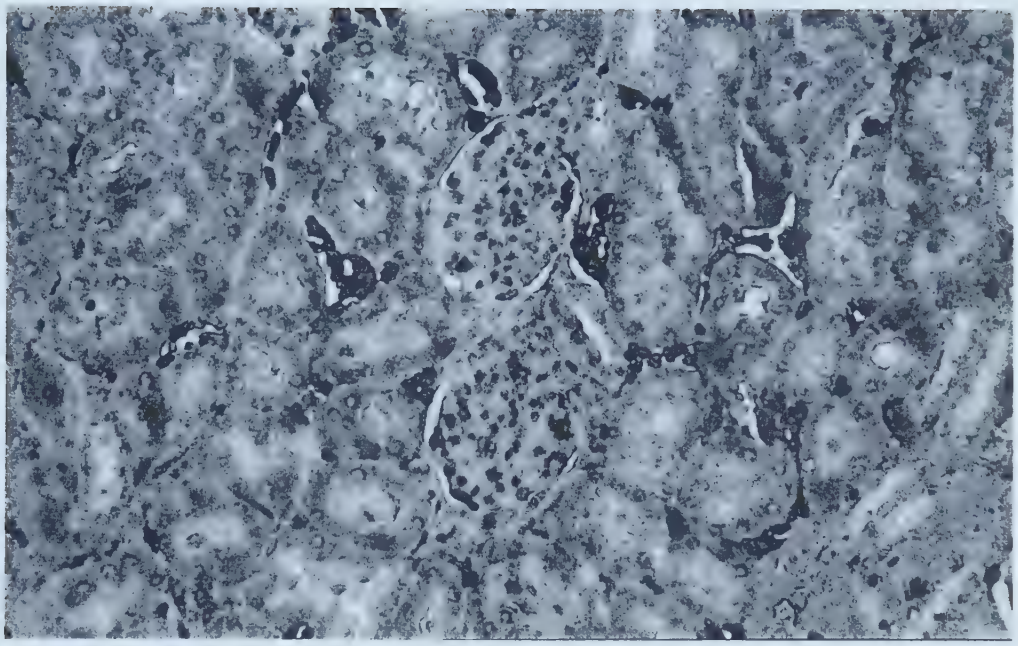


Figure 3.3. Autoradiograph of immunoprecipitations analysed by SDS-PAGE showing Class I association *in vivo* is predominately with D^b in NOD/Ltj and NOR/Ltj mice. Eight week old NOD and NOR mice were fasted overnight and then fed $1x10^7$ cpm (≈15µg) of ^{125}I labeled Bovß2-M by gavage*. Animals were sacrificed 4 hours later, splenocytes prepared and lysed, and class I molecules immunoprecipitated from separate aliquots using monoclonal antibodies specific for K^d (SF1.1.1) and D^b (B22-249).

*gavage feeding can result in the sporadic absorption of relatively larger quantities of Bovß2-M than are seen with voluntary feeding (see Chapter 2).

A.



B.

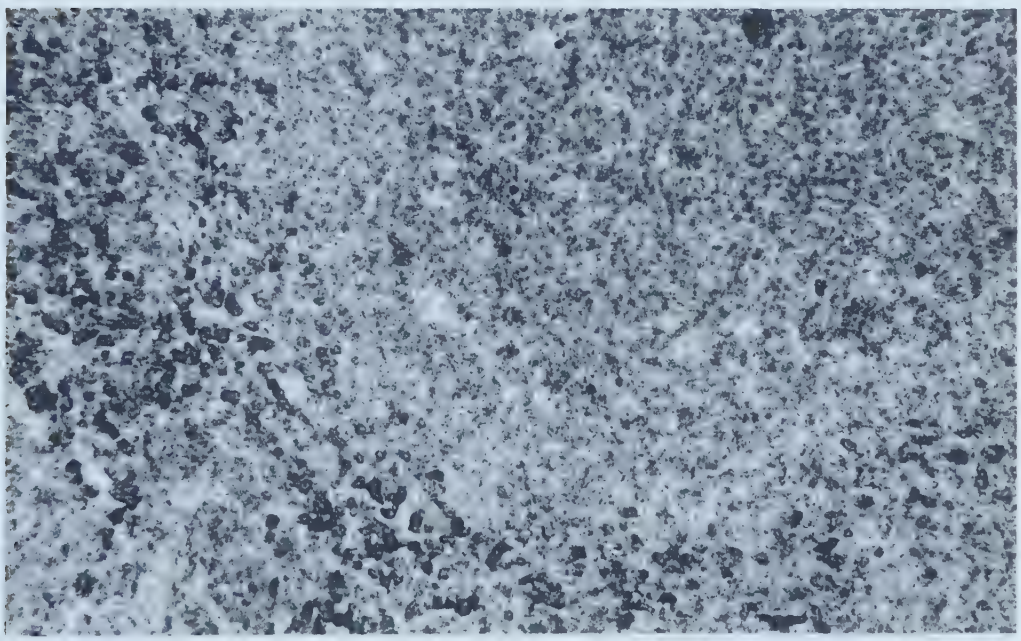
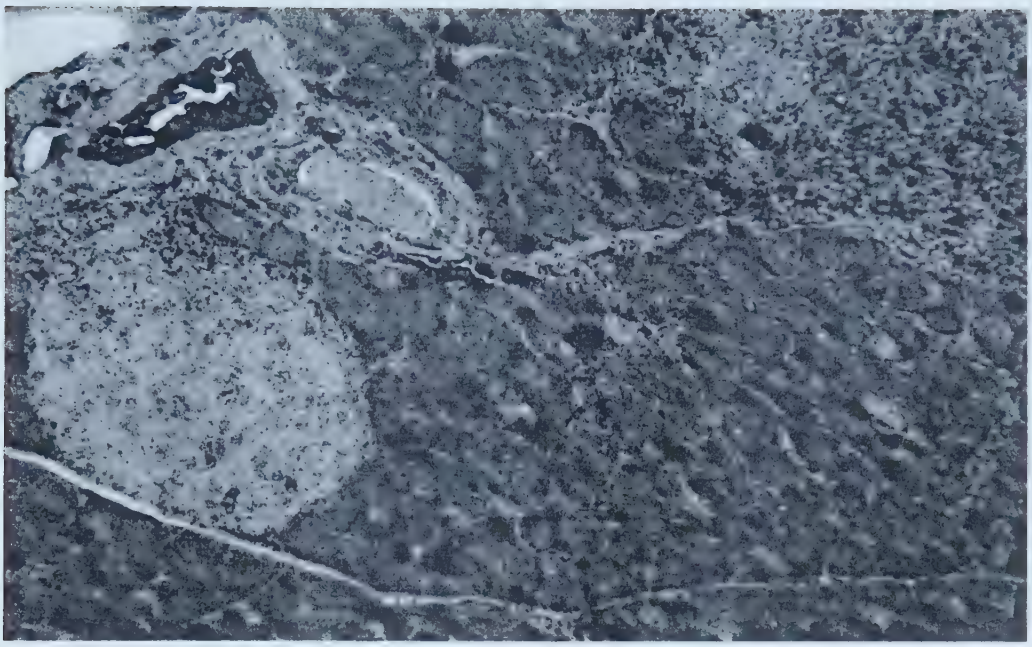


Figure 3.4. Deposition of Bovß2-M in various tissues following IV injection of ^{125}I labeled protein into a 12 week old NOD/Ltj male. The animal was sacrificed 4 hours post-injection, and various tissues sectioned and subjected to in situ autoradiography and H and E staining.

A) Kidney - 2 week exposure, x160 B) Spleen - 2 week exposure, x 160.

Sections show preferential deposition of labeled protein (i.e. dark areas with dense silver grains) on the surface of endothelial cells, erythrocytes and some splenocytes.

C.



D.

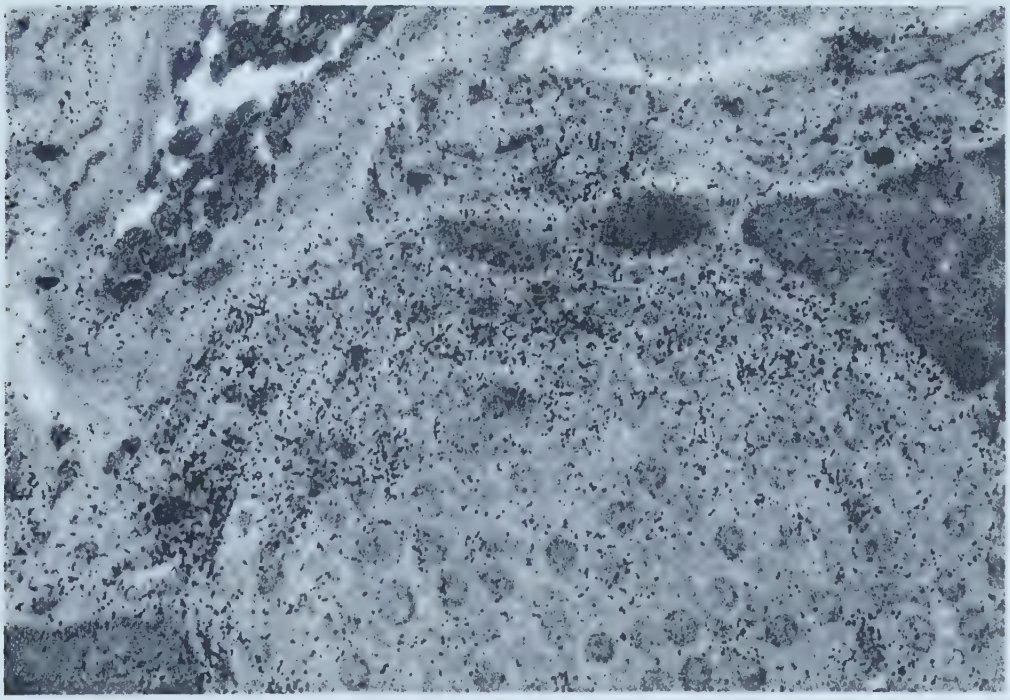
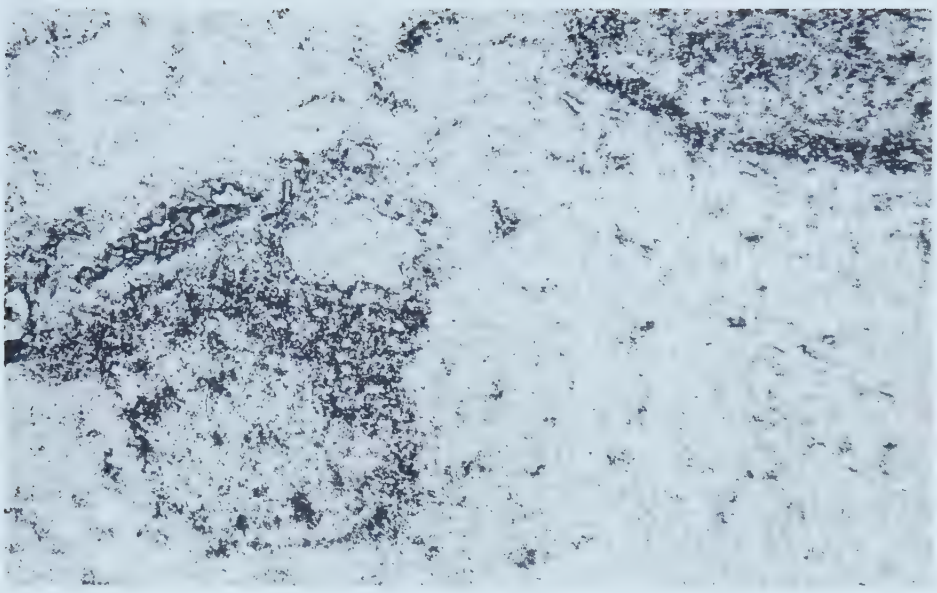


Figure 3.4. (continued) C) Pancreas - showing a relatively normal islet together with a blood vessel and duct (left) and a more inflamed islet (upper right; shows mononuclear cell infiltrate). (2 wk. exp. x 160) D) Pancreas, higher magnification of same field as above, showing detail of left islet and vessel (2 wk. exp. x 500)

E.



F.

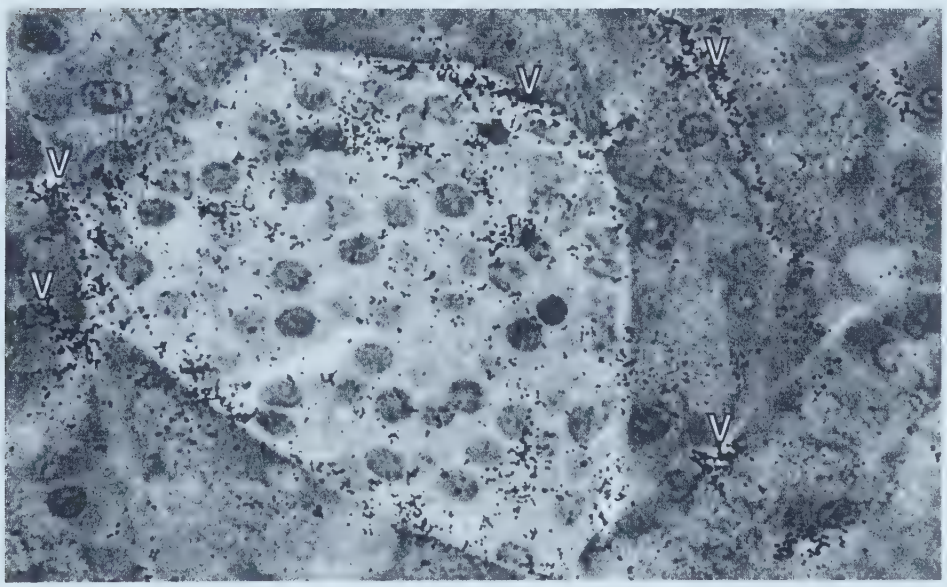


Figure 3.4. (continued) E) Pancreas, unstained, very similar field as in C), showing preferential deposition of Bovß2-M in the islets versus exocrine pancreas. (6 week exposure, x 160) F) Detail of normal islet, showing Bovß2-M deposited in vessels surrounding islet (**V**), as well as within the islet itself. (6 week exposure, x 640)

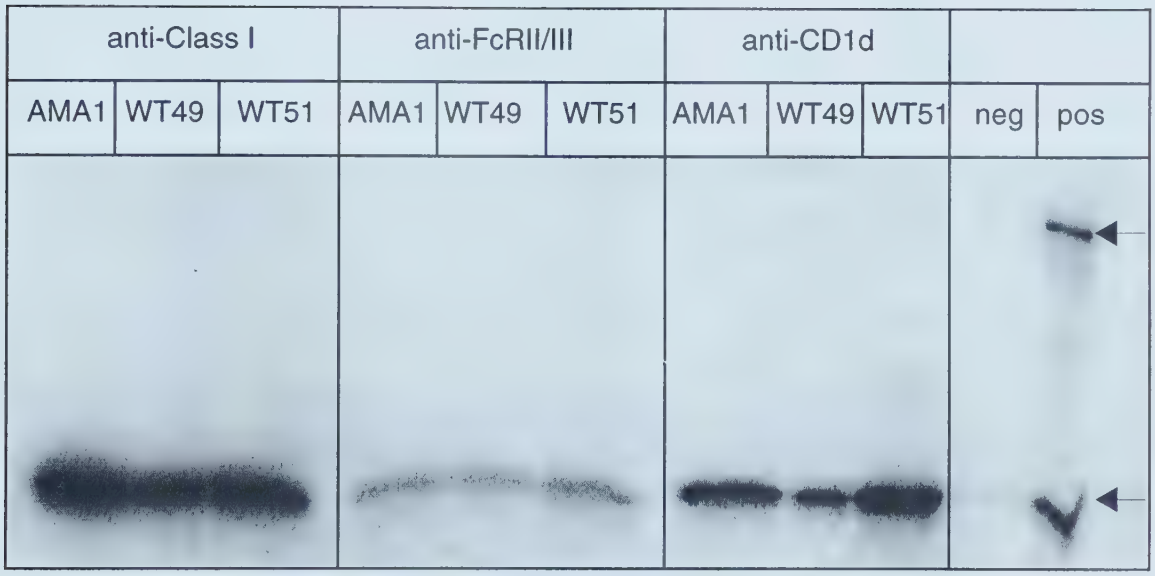
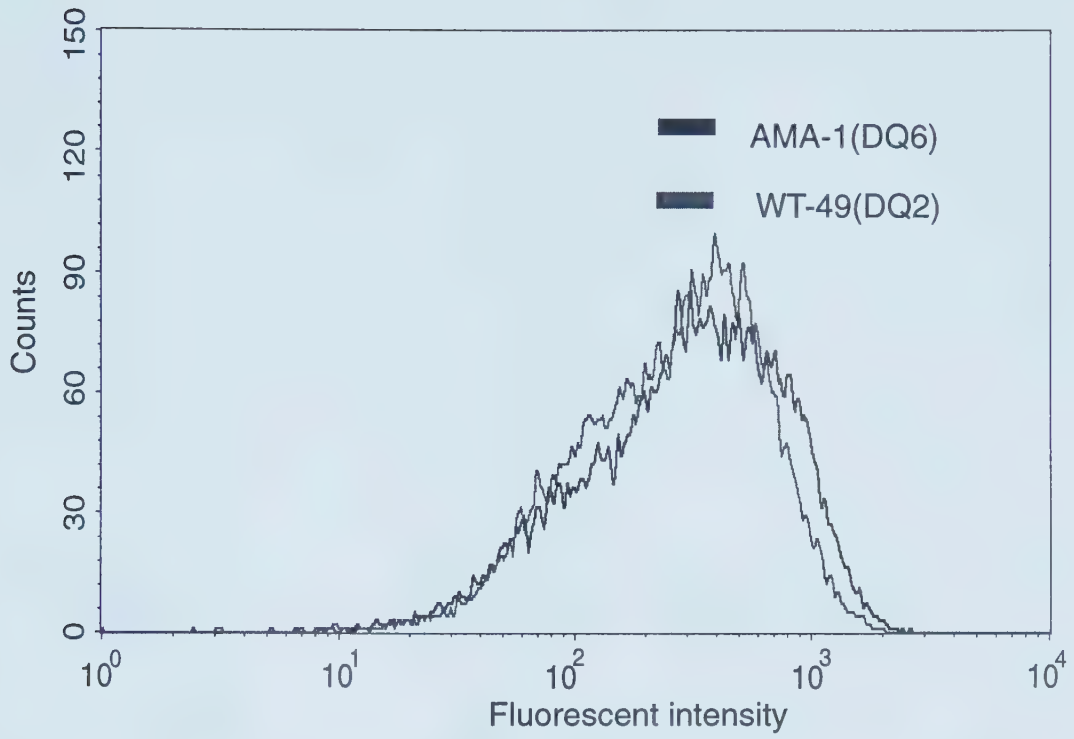


Figure 3.5 Radiolabeled Bovß2-M co-immunoprecipitates with human class I MHC, FcR II/III, and CD1d following incubation in vitro with EBV transformed human B cell lines. Human typing cells were incubated at 37°C / 5% CO_2 for 4 hours in protein free hybridoma medium (PFHM-II) containing ^{125}I-labeled Bovß2-M (1µg/mL). Cells were then washed x 3, lysed, and cell lysates precleared with an isotype matched control monoclonal antibody and protein A sepharose. Immunoprecipitations were performed on separate aliquots of the pre-cleared lysate, using monoclonals W6-32 (anti-Class I), 2.4G2 (anti FcRII/III), and 1H1 (anti-CD1d). Negative control (neg) is an isotype matched monoclonal antibody used at equivalent concentration after the preclearing step. The upper and lower arrows denote the size of BSA and Bovß2-M standards respectively. In these experiments three different human B cell lines were used: AMA1(DQ6), WT49(DQ2) and WT51(DQ8).

A.



B.

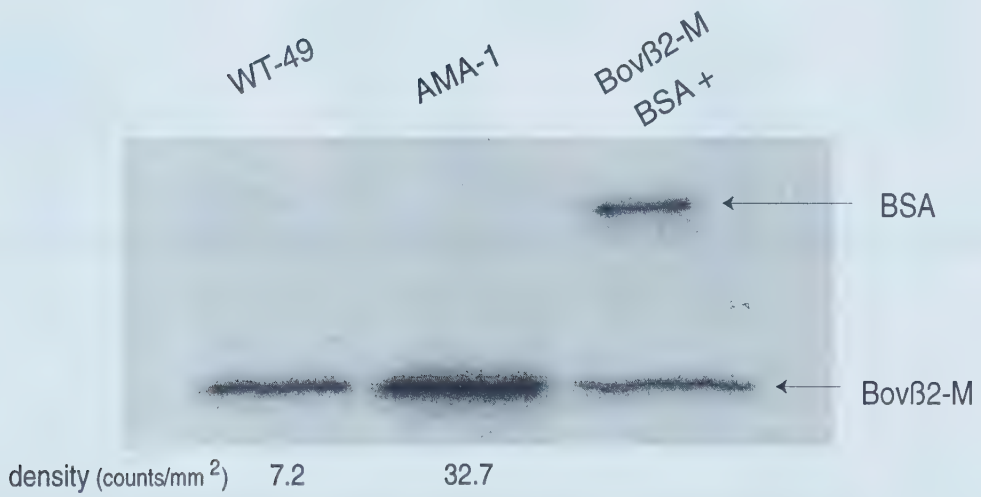


Figure 3.6. Comparison of cell surface expression of Class I MHC and relative amount of Bovß2-M bound by Class I for equal numbers of the human typing cells WT-49 and AMA-1. **A.** Class I staining of WT-49 and AMA-1 cells with W6-32/FITC (stains all class I haplotypes). **B.** Immunoprecipitations (with McAb W6-32) of WT-49 and AMA-1 after incubating cells for 4 hours with ^{125}I Bovß2-M (1µg/mL) in PFHM-II at 37°C. The AMA-1 cells appear to have accumulated considerably more class I associated Bovß2-M than an equivalent number of WT-49 cells, even though both cell lines express similar levels of class I. Control proteins are in the right hand lane.

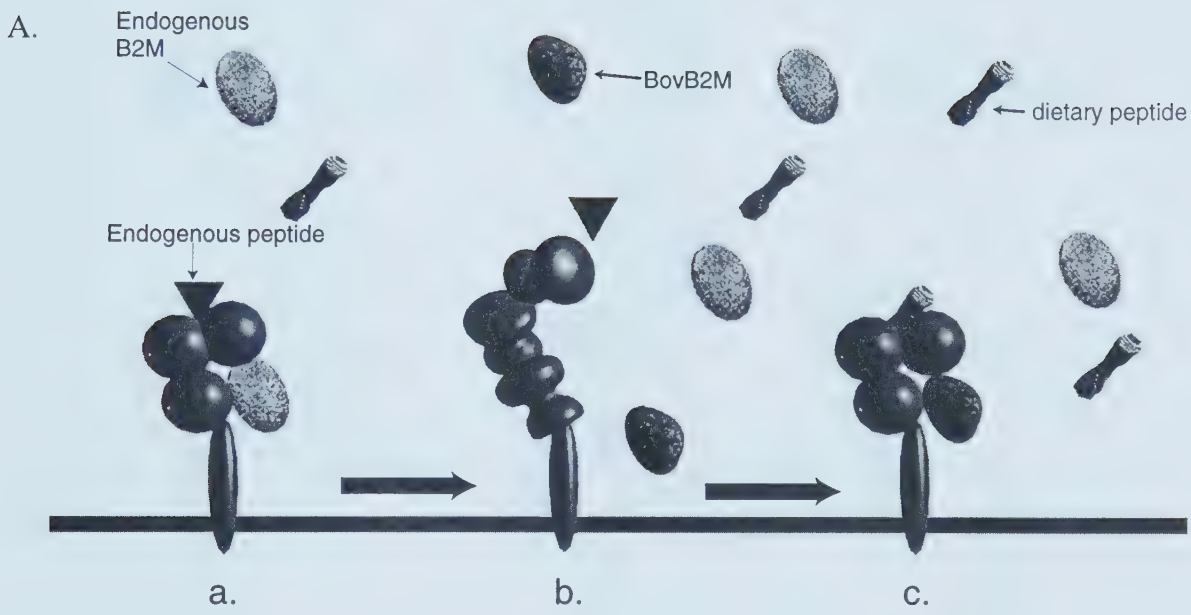


Figure 3.7. Potential models to explain the adjuvant role of Bovß2-M in stimulation of T cell responses.

A. Exogenous Bovß2-M promotes in vivo loading of dietary or other free peptides at the cell surface.

a. Conformationally intact MHC class I with endogenous ß2-M peptide (to which T cells are tolerant).

b. Loss of endogenous ß2-M and endogenous peptide, alpha chain unfolds and is destined to be degraded.

c. Association of exogenous Bovß2-M with unfolded alpha chain rescues molecule and promotes loading of dietary or other free peptides at the cell surface; conformationally intact MHC is regenerated, but bound to a novel peptide which does not arise from within the cell (T cells may not be tolerant).

B.

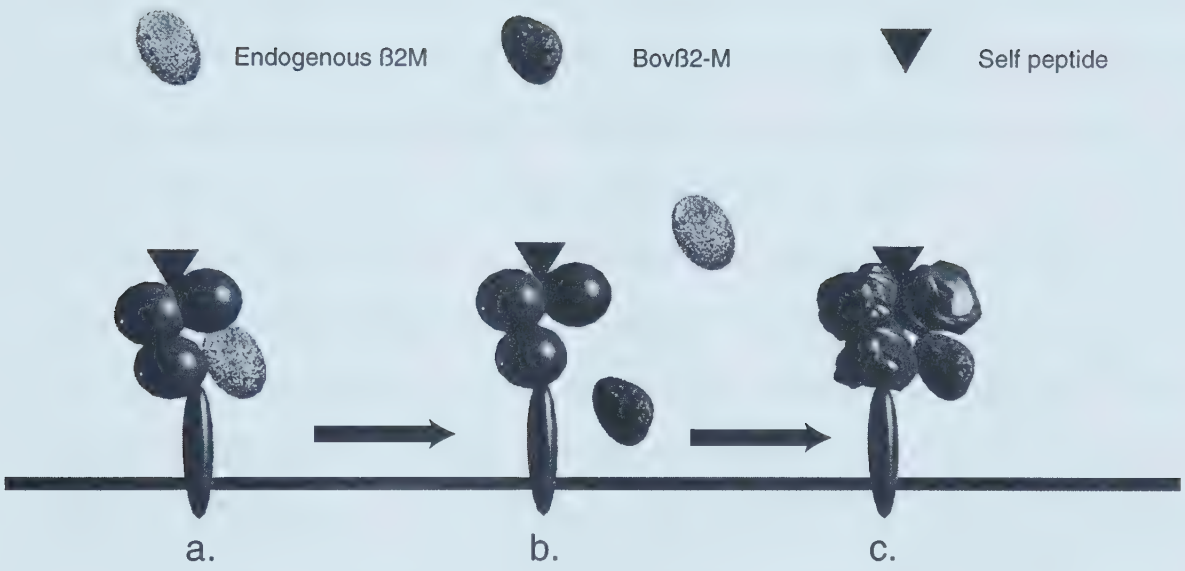


Figure 3.7. (continued).

B. Binding of exogenous Bovß2-M induces a conformational change in the class I MHC; T cells now respond to endogenous peptides loaded in the altered class I.

a. Conformationally intact MHC class I with endogenous ß2-M and peptide (to which T cells are tolerant).

b. Loss of endogenous ß2-M; alpha chain remains folded with endogenous peptide bound for a brief period.

c. Rapid association of exogenous Bovß2-M with alpha chain and endogenous peptide induces a conformational change in the alpha 1 and / or alpha 2 domains ; these are now recognized by T cells as foreign although an endogenous peptide remains bound to the groove.

CHAPTER 4

INTRAVENOUSLY INJECTED RADIOLABELED BOVINE ß2-MICROGLOBULIN HAS A LONG CIRCULATORY HALF-LIFE WHICH DECREASES ABRUPTLY EIGHT HOURS POST-INJECTION IN NOD/LtJ BUT NOT IN NOR/LtJ MICE

A. Abstract

Bovine ß2-microglobulin is a small, acid and protease-resistant protein which is found in cows' milk. In previous work we have shown that a portion of orally ingested bovine ß2-microglobulin will make its way intact into the circulation of mice, where it can heterodimerize with mouse class I MHC and CD1 molecules, with possible immunological consequences. The purpose of the present study was to determine the half-life of bovine ß2-microglobulin in the mouse circulation. Diabetes prone NOD/Ltj and genetically similar but diabetes resistant NOR/Ltj mice were given an intravenous injection of ^{125}I-labeled bovine ß2-microglobulin, and plasma samples were collected at intervals thereafter and analyzed for the presence of the intact labeled protein using SDS-PAGE and quantitative phosphorimaging. During the first 8 hours post-injection the concentration of the radiolabeled protein fell in an identical fashion in both strains of mice, with roughly 80% of the protein disappearing within 30 minutes, and the remainder declining exponentially, but with a surprisingly long half-time ($T_{1/2}$ = 30.1 h). From 8 to 50 h post-injection, however, the rate of protein disappearance was abruptly increased, but diverged markedly between the two strains of mice, with the $T_{1/2}$ decreasing slightly to 23.2 h for the NOR mice, and more markedly to 7.2 h for the NOD mice. We hypothesize that the accelerated disappearance of bovine ß2-microglobulin beginning 8 h after injection is the result of immune activation caused by injection of the heterologous protein itself, and that the level of immune activation occurring is much greater in the diabetes-prone NOD/Ljt mice than the

diabetes-resistant NOR/Ljt mice. Since both strains of mice would have encountered bovine ß2-microglobulin (i.e. cows' milk proteins) in their diet, this suggests that NOD mice may be more prone to develop immune responses against dietary antigens.

B. Introduction

In comparing a variety of countries, per capita consumption of cows' milk has been shown to be positively correlated with the incidence of type I or autoimmune diabetes (82,83). In previous studies we have shown that orally ingested bovine ß2-M (a protein present in cows' milk) can be absorbed intact into the circulation of mice and can pair with endogenous MHC class I molecules, and because we believe that this may have immunological consequences, we were interested to know the circulatory half-life of bovine ß2-M in mice. Kinetic models for the renal catabolism of ß2-M have been established for: 1) human ß2-M in humans, 2) rat ß2-M in rats, and 3) human ß2-M in rats (19,89,96). Kinetic models for 1) and 2) follow a two exponential model, with circulatory half-lives of less than 5 minutes for the first exponential decay, and 2.1 - 10.2 h for the second exponential decay. Kinetics for 3) are best matched by a three exponential model due to the presence of a rat plasma factor which binds human ß2-M. In spite of similarities, no single mathematical model has been developed which can account for all available *in vivo* data from humans and rats. Since we were interested in the catabolism of bovine ß2-M in mice, we felt that it would be difficult or impossible to predict how existing kinetic models might apply to this particular species combination. Therefore we determined to answer the question experimentally using two different strains of mice: 1) NOD/Ltj, which spontaneously develop autoimmune diabetes at 16-20 weeks of age, and 2) NOR/Ltj, which do not develop diabetes, although they express the same MHC molecules as NOD, and are genetically very similar. We believe this represents the first report on the behavior of circulating bovine ß2-M in mice.

Like many other low molecular weight proteins, endogenous beta 2-microglobulin (ß2-M) is catabolized primarily in the kidney. In humans and rats ß2-M has been shown to be filtered freely through the glomerular membrane, with a sieving coefficient of 0.97 (97). It is then re absorbed and a portion of the protein degraded in the proximal tubules, with transport occurring via both high and low affinity interactions with molecules of the brush-border membrane (97). It is well known that an increase in plasma and urinary concentrations of ß2-M occur following loss of renal function, with minor losses of glomerular/tubular function resulting in relatively large increases in ß2-M levels in the plasma/urine respectively.

C. Materials and methods

Mice

NOD/Ltj and NOR/Ltj mice (male, 8 weeks) were obtained from local breeding colonies maintained by the University of Alberta Health Sciences Laboratory Animal Services (HSLAS), originating from Jackson Laboratories (Bar Harbor, ME). All procedures involving animals received the approval of the local Health Sciences Animal Welfare Committee, and experiments were carried out under consultation with the veterinary and technical staff of the HSLAS.

Proteins and radiolabeling

Bovß2-M was isolated from bovine colostrum via the method of Groves and Greenberg (86). Protein was labeled by a tyrosine oxidative iodination. Bovß2-M (500µg) or BSA (500µg) in 500µL of PBS pH 5.6 was added to glass tube (12mm x75mm Fisher Scientific, U.S.A.) with one IODO-BEAD® per tube (Pierce, Rockford, IL). 5mCi $Na^{125}I$ (IMS.300 Amersham, Arlington Heights, IL) in a vol of 9µL was then added to the solution. The iodination reaction was allowed to proceed at 20˚C for 7 min. The reaction was terminated by removing the liquid with a pasteur pipette from the glass tube

and the Iodobead and then passed through a size exclusion column to separate free ^{125}I from labeled protein. The column consisted of a 10 ml polypropylene syringe with 5 ml of wet volume G-10 sephadex equilibrated in PBS pH 5.6. Fractions (0.5 ml) were collected and 1µL of each fraction counted with a LKB Wallac 1275 gamma counter. Peak fractions were combined and TCA precipitable counts determined as follows. 0.5 ml of 1% BSA (w/v) in PBS pH 5.6 was added to four tubes and 25 µl pooled fractions added to each tube. To two tubes 0.5 ml of PBS pH 5.6 was added, and to the remaining tubes 0.5 ml of 10% TCA in PBS pH 5.6 was added. Tubes were placed on ice for 15' and spun 500 x G at RT for 15'. 20 µl of supernatant from the tubes was removed and counted. Protein associated counts ranged from 95-98% and specific activity was determined by absorbance of solution with OD at 280nm. Bovß2-M specific activity was ≈ $1x10^6$cpm/µg

Injections

Penile IV injections of $1x10^6$ cpm of ^{125}I-labeled Bovß2-M (1µg) were performed on anaesthetized 8 week old male NOD/Ltj (n=3) and NOR/Ltj (n=4) mice.

Collection of blood and plasma samples

Time course studies were performed by taking 20 µL whole blood samples from the tail tip into heparinized glass capillary tubes at various times up to 48 h post-injection. Blood was expelled from the capillaries into 250µl eppendorf tubes with the aid of an adapter attached to a P200 Gilson pipetman. Cells were separated from plasma by spinning samples in a microfuge at 3000 x G for 30 s. Plasma was removed from cells, placed in a fresh 250µl tube, and stored at -20°C. Aliquots (1µl) of fresh or frozen plasma were analyzed by SDS-PAGE.

SDS-PAGE

All SDS-PAGE gels were cast and run in the BioRad (Hercules, CA) MiniGel™ apparatus, using a 5% Tris/Glycine/acrylamide stacking gel and a 15% separating gel. Samples containing SDS loading dye were heated at 90°C for 5' and loaded immediately onto the gels (5-20µl/lane), which were run at aconstant current (25 mA) for 50-60 min. Gels were fixed and stained for 15 min in a solution of 50% methanol, 10% acetic acid, 0.5% comassie blue G250; destained for 60 min in 10% methanol, 10% acetic acid; and then dried under vacuum at 80°C for 30 min. Dried gels were placed on a BAS-IIIs™ phosphorimager plate (Fuji®, Japan) for 3 days with a serial dilution ^{125}I Bovß2-M standards.

Densitomitry and Calculations

Densitomitry was performed using MacBas ™ software (Fuji®, Japan). Densitomitry results from the time course were related to initial injection values using the serial dilution standards of known counts of the same ^{125}I Bovß2-M used in the injections. Time zero value for Bovß2-M plasma level was calculated as the total protein associated counts injected IV divided by the estimated volume of blood (14% (v/w) of body weight). Mice body weights were in the range of 18.1-21.8g. Individual mice Bovß2-M plasma values were normalized against 30 min value, allowing comparison between individual mice.

Statistics

Densitomitry values once normalized between mice, were entered into Cricket Graph (version 3.1) (Computer Associates International, Inc) software. Once tabulated, the average values were calculated for each time point and error bars assigned to these values to denote variance between the mice in the group. A "best fit" curve was obtained by using

software to determine logarithmic equations that matched the data with the highest correlation value (r) between calculated and observed results. Equations were of the form:

$$Cp(t) = A * e^{-(alpha * t)} + B * e^{-(beta * t)}$$

Cp(t) = plasma drug concentration at time "t"
A = Intercept of first exponential term (process)
B = Intercept of second exponential term (process)
alpha = -slope of first exponential term
beta = -slope of second exponential term

[By convention, the slowest exponential process is placed as the second term]

Ke, the elimination rate constant can be defined as the fraction of Bovß2-M in the plasma that is eliminated per unit of time.

$$Cp(t) = Cp(0) * e^{-(Ke * t)}$$

Elimination half-life is the time required for the amount of Bovß2-M (or concentration) in the plasma to decrease by half.

$$\text{Half-life } (t_{1/2}) = \ln 2 / Ke = 0.693/Ke$$

D. Results

For the initial 8 h post injection (figure 4.1) the serum Bovß2-M in NOD and NOR mice disappeared with kinetics which closely resembled the two exponential model used to describe the behavior of human ß2-M in anaphoric rats (98). An initial rapid drop of Bovß2-M plasma levels occurs during the first minutes post IV injection, corresponding to diffusion into the extraplasmic compartment (99), followed by a remarkably slow disappearance of free Bovß2-M from the plasma ($T_{1/2}$ =30.1.h). During this period of

time the plasma half life of Bovß2-M is essentially identical between NOD (r=0.520) and NOR(r=0.812) mice. However, over the subsequent time period of 8 - 48 h post-injection, the half life of Bovß2-M in NOD and NOR plasma change and become significantly different (figure 4.2), with the NOR $T_{1/2}$ dropping slightly to 23.2 h (r=0.997) while the NOD $T_{1/2}$ drops three fold to 7.2 h (r= 0.995).

E. Discussion

Kinetics of disappearance of Bovß2-M in the first 8 hours post-injection

In the first 8 h post IV injection both NOD and NOR strains have remarkably similar Bovß2-M plasma kinetics. After injection, an initial rapid exponential drop in plasma levels ($T_{1/2} \approx 13$ min) was observed, characteristic of all previous ß2-M kinetic studies. This drop is indicative of the loss of protein from plasma as it passes into extraplasmic compartments (cellular and extracellular matrix associated). Once an equilibrium is reached between the plasmatic and extraplasmic compartments, the rapid drop in Bovß2-M plasma level halts and a second more gradual exponential loss is observed with a $T_{1/2}$ = 30.1 h. This value is considerably higher than any previously observed $T_{1/2}$ from studies of human and murine ß2-M, where values ranged from 2.1 - 10.2 h (19,89,96). The cause of the increased $T_{1/2}$ is unknown at this time. This is the first measurement of Bovß2-M $T_{1/2}$ in murine plasma, and the kinetics of disappearance of murine ß2-M have yet to be determined in mice. It is possible that the high $T_{1/2}$ is a result of a murine physiological trait. To resolve this question a similar study must be performed with the murine ß2-M.

However, Bovß2-M is known to have several unique characteristics in relation to human and murine ß2-M. Bovß2-M has a time dependent, concentration and pH dependent ability to aggregate irreversibly into multimers. This phenomenon occurs even at low concentrations in vivo. No aggregation of free human ß2-M at similar concentrations has been noted, although low levels of dimeric human ß2-M have been found at high protein concentrations (non physiological), as have glycosylated multimeric human ß2-M in

amyloid deposits of patients with renal failure (100). Bovß2-M has also been found to be highly insoluble at physiological pH, and once in solution at this pH range to have high non-specific association with other high molecular weight proteins such as immunoglobulins. These characteristics may contribute alone or in combination to the unusually long $T_{1/2}$ observed for Bovß2-M in mice.

We have shown previously that in vivo extraplasmic deposition of Bovß2-M occurs differentially into the cellular compartments via interaction with mouse class I heavy chains. Both NOD and NOR strains possess identical class I heavy chains (D^b & K^d), but have different alleles of murine ß2-M which differ at one amino acid (D or A at position 85). The D^b heavy chain is known to form a relatively unstable heterodimer with the murine ß2-Ms, and the endogenous ß2-M is readily replaced by Hum or Bovß2-M. D^b heavy chain is also known to be present on the cell surface at significant levels in a ß2-M "free" unfolded form allowing for even greater Bovß2-M uptake. Heterodimerization of Bovß2-M with murine class I in vivo is known to occur at significant levels and would prevent passive filtering at the glomerulus, extending the observed $T_{1/2}$. Similarity between an anaphoric rat model (98) and our results suggests that the kidney may not play the same predominate role in Bovß2-M catabolism as it does for the catabolism of endogenous ß2-Ms.

The $T_{1/2}$ of Bovß2-M in mice may also be the result of other interactions in the kidneys. Variations in the charge of low molecular weight proteins are known to affect their glomerular filtration rate, with the passage of negatively charged proteins being hindered(101). There are significant differences in the pI of ß2-M from different species, as well as differences between the various isoforms of human ß2-M frequently seen in amyloid deposits (see Chapter 1)(102). The variation of charge between these highly homologous proteins may be involved in differential filtering in the kidney, allowing ß2-M from one species to stay in the circulation longer than others. Proximal tube reabsorption of rat and human ß2-M is believed to require conformationally intact proteins, and to involve very specific binding to receptors. Studies using albumin to compete with high affinity

ligands for ß2-M in the brush border membrane are indicative of this specificity (19). This is another potential site for differential reabsorption and/or catabolism of Bovß2-M compared to other ß2-Ms.

Plasma binding factors

A two exponential model is sufficient to describe the plasma kinetics of most small molecular weight proteins in circulation (99). However, to describe human ß2-M in the plasma of human subjects, a simplified three exponential model is required to account for the more complex kinetics and longer plasma $T_{1/2}$ seen in patients undergoing long term dialysis (102). The same requirement for a three exponential model is also seen in normal rats(89). In rats, the three exponential model accounts for the presence of a 40-80kDa ß2-M binding protein in plasma which prevents the passage of ß2-M through the glomerular filter and thus hinders its renal catabolism (19). Polyclonal IgG auto-antibodies against human ß2-M have also been described in healthy humans (103). High levels of these autoantibodies have been found in patients with various autoimmune and chronic inflammatory diseases (104,105). The effect of these autoantibodies on the catabolism of ß2-M has not been addressed (98). It is possible that anti-ß2-M IgG is responsible for the appearance of the third exponential phase and the increase of plasma $T_{1/2}$ seen in long term dialysis patients, which conceivably could have higher levels of circulating anti ß2-M antibodies in response to unusually high levels of circulating and amyloidic ß2-M. Antibodies against Bovß2-M in mice may also be involved in preventing renal catabolism, and thus account for the long half-life observed.

Kinetics of disappearance of Bovß2-M in the subsequent 8 to 48 hours post-injection

As the plasma kinetics for NOD and NOR strains were identical for the initial 8 hours post IV injection we did not expect to see them differ later on. We found that in both strains, after 8 hours the plasma Bovß2-M levels followed a third and final exponential

slope, but that this differed significantly between the two strains. The NOD males exhibited a dramatic increase in Bovß2-M loss, with new $T_{1/2}$ value stabilizing at 7.2 h for the remainder of the study. NOR males exhibited a more minor increase in Bovß2-M loss, with the $T_{1/2}$ value dropping from 30.1 h to 23.2 h.

There are several possible explanations for this late change in the rate of disappearance of Bovß2-M in the NOD and NOR strains. The first is an increased cellular uptake of Bovß2-M, which is clearly greater in the NOD strain than in the NOR. Previous studies showed that in the rat, tissue deposition was roughly related to macrophage content of the tissue(89), presumably related to the high levels of class I MHC on these cells. Comparisons of tissue deposition between the two strains was not performed in this series of experiments, but it would be interesting to see if at 8 h post-injection there was a higher level of macrophage activation (as reflected by increased class I expression) in NOD mice than in NOR.

From unpublished *in vitro* observations (D. Denny, personal communication) D^b is known to form a heterodimer with the "B" allele of Murine ß2-M (NOR) more readily than with the "A" allele (NOD). The effects of the different murine ß2-M alleles on the relative stability of other class I and class I-like molecules which require ß2-M association for function are unknown. This may also play a role in the long term $T_{1/2}$ differences observed between NOD and NOR strains. Perhaps Bovß2-M is more able to replace the "A" allele ß2-M on D^b, or other molecules, allowing (for a given level of immune activation) greater cellular deposition and concurrent loss of Bovß2-M from the plasma.

Perhaps the most intriguing result from our kinetic study was the abrupt change in plasma Bovß2-M levels 8 hours after injection. It is at this time that natural killer cells (Nk1+) and macrophages could be expected to be maximally activated in response to foreign proteins. Bovß2-M has known adjuvant properties which could stimulate such immune activation. If these cells are involved, it could represent an important difference between the two strains, with respect to their capacity to mount an immune response

against a protein similar to self. The rapid drop of Bovß2-M seen in NOD mice after 8 hours could be an indication of a predisposition of their cellular immune responses towards Bovß2-M, a response not seen to the same degree in the disease resistant, MHC matched, NOR strain.

Our study was not intended to be a complete metabolic study of Bovß2-M in mice. It was simply our intention to carry out observations for plasma half lives, similar to those already done in the rat, as a basis for other metabolic studies. However it has uncovered a hitherto unknown difference between metabolism of ß2-M in the NOD strain (mouse model for IDDM) and the genetically similar, diabetes free, NOR strain. This difference warrants further study and could provide important clues to the role of altered class I restricted responses in development of autoimmune diseases. Further comprehensive studies should now be performed to determine the predominate sites of Bovß2-M catabolism, and to examine the difference between the *in vivo* kinetics of Bovß2-M between the NOD and NOR strains.

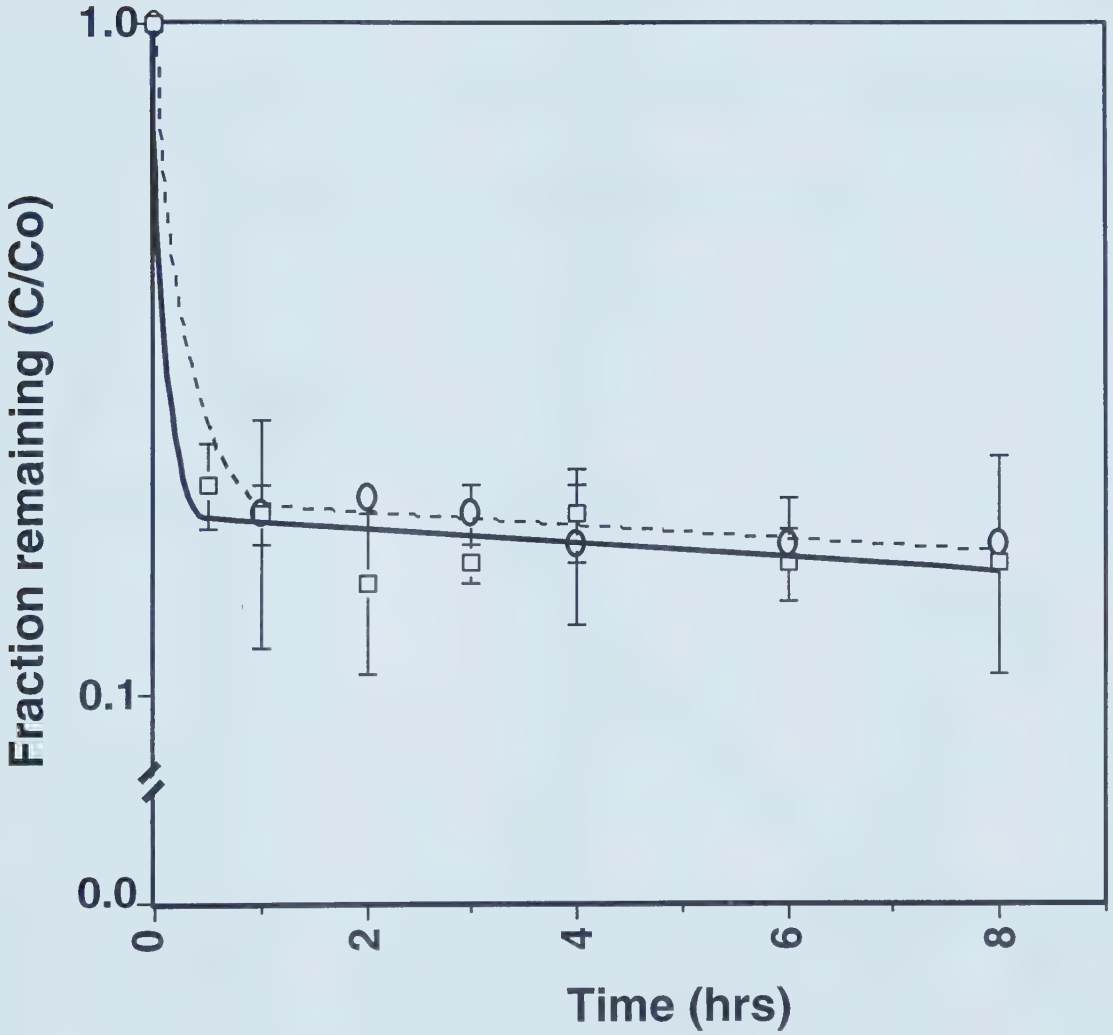


Figure 4.1. Short term (≤ 8 h) kinetics for the disapperance of intact Bovß2-M from the plasma of NOD/Ltj (n = 3) and NOR/Ltj (n = 4) male mice. An exponential decay model for the intermediate period post injection (i.e. 0.5 - 8 h) gives a plasma half life of 30.1 h.

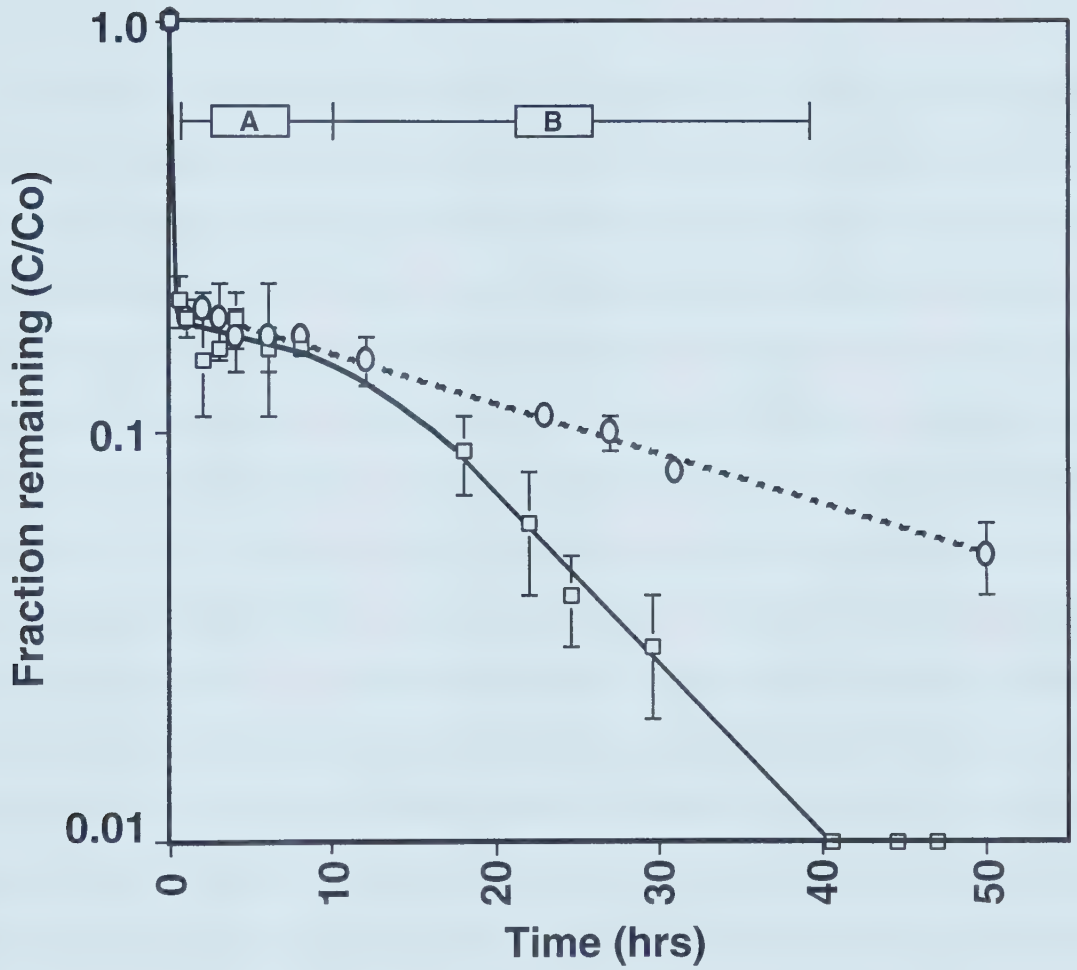


Figure 4.2. Kinetics for the disapperance of intact Bovß2-M from the plasma over the longer term post injection (i.e. 8 - 48 h.) in NOD/Ltj (n=3) and NOR/Ltj (n=4) male mice. Two different exponential decays are observed, corresponding to period A (0.5 to 8 h. post injection; see figure 4.1) and period B (8 - 48 h. post injection). For period B the half life of Bovß2-M is 7.2 h. in NOD/Ltj mice (r = 0.995) and 23.2 h. for NOR/Ltj mice (r = 0.997).

CHAPTER 5

THE BOVINE ß2-MICROGLOBULIN PRESENT IN COMMERCIAL COWS' MILK IS CONFORMATIONALLY INTACT AND CAN PAIR WITH MOUSE CLASS I MHC MOLECULES

A. Abstract

Bovine ß2 microglobulin (Bovß2-M) is a small, acid stable 11.5 kDa protein consisting of a single immunoglobulin domain, which is present at relatively high levels in cow's milk. In theory, orally ingested Bovß2-M can be absorbed systemically and pair specifically with human MHC class I heavy chains to affect the regulation of allergic or autoimmune responses, but only if the ingested Bovß2-M is conformationally intact. We conducted the present series of experiments to test whether or not conformationally intact, 'biologically active' Bovß2-M is present in commercially available homogenized and pasteurized (72°C x 15 s) dairy products. Using a sensitive in vitro cellular binding assay coupled with monoclonal antibody staining and fluorescent activated cell scanning (FACS) analysis, we were able to show that conformationally intact Bovß2-M capable of pairing with mouse class I MHC heavy chains is present in commercial milk. Further, we used our assay to show that some biologically active Bovß2-M remains in milk even after heating at 90°C for 120 min. Western blots indicated that in addition to milk, intact Bovß2-M is present at relatively high levels in all dairy products examined, including cheese, ice cream, and yogurt, whereas it is present at a much lower levels in raw or cooked beef. The remarkable thermal stability of Bovß2-M suggests that simple heat treatment will not be a practical method to destroy the biological activity of this bovine immune globulin present in dairy products.

B. Introduction

There are two major immune globulins in cow's milk: antibodies (primarily IgG) and bovine ß2-microglobulin (Bovß2-M). ß2-microglobulin is the smallest member (11.5 kDa) of the immunoglobulin super-family, consisting of a single disulfide-linked immunoglobulin domain, and it is found free in all bodily fluids (average concentration 1.0-1.5 µg/ml), as well as in strong non-covalent association with MHC class I heavy chains. Bovine IgG and Bovß2-M are both known to be stable at low pH, and both are relatively resistant to proteolysis. In humans, intact ^{15}N labeled bovine IgGs have been found at the terminal ileum after oral consumption of ^{15}N labeled milk proteins, proving that IgG can pass through the stomach and small intestine without necessarily being digested(94). Given the similarities between the structures and biochemical properties of bovine IgG and bovine ß2-M, it is highly likely that the latter molecule can also pass through the human digestive tract without necessarily being digested.

Similar to bovine IgG, Bovß2-M is especially abundant in bovine colostrum (150 µg/ml), but it is also present at relatively high concentrations in cow's milk (30 µg/ml) (86). Likewise, human ß2-M is most highly concentrated in colostrum, but it is also abundant in human milk, again in parallel with maternal antibodies (106). Maeda *et al.* showed that when nursing mothers ingested cows' milk in the perinatal period, detectable levels of bovine IgG appeared in their colostrum within 7 h (107), suggesting that bovine IgG not only makes its way intact through the stomach and small bowel, but also that the intact xenogeneic IgG is efficiently absorbed, at least in lactating mothers. Perhaps relevant here is the parallel observation in mice that a higher systemic uptake of intact dietary proteins occurs in lactating animals, and that these absorbed proteins are concentrated in the mammary glands(108). Interestingly, in formula fed infants bovine IgGs have been frequently implicated as a causative agent in serious gastric conditions such as chronic colic (95), and in extreme cases, mothers breast feeding allergic infants have been required to remove cows' milk and dairy products from their diet (H. Pabst, personal communication).

To our knowledge, the question of whether or not Bovß2-M is implicated in cows' milk allergies in either infants or adults has not been examined to date.

Within cells, ß2-M expression is necessary for the optimal processing and intracellular transport of the MHC class I heterodimer after synthesis in the endoplasmic reticulum (20,21). Endogenous class I associated ß2-M and free ß2-M are known to exchange at the cell surface in vitro (22) and in vivo (23). Both human and bovine ß2-M are known to efficiently exchange with murine ß2-M on murine class I heavy chains (24,25), and particularly with D^b which is found on the mouse cell line EL4 used in the present series of experiments. In addition, we and others have shown that Bovß2-M can exchange with human ß2-M on human class I heavy chains.

As stated above, the biochemical properties of Bovß2-M suggest that it would be relatively resistant to digestion in the gastrointestinal tract, and we have previously shown in adult mice (but not yet in humans) that a small portion of orally ingested Bovß2-M is absorbed intact into the circulation. Because Bovß2-M is likely capable of making its way through the gastrointestinal tract and into the circulation intact in humans, and because the molecule can specifically pair with and potentially modify the structure of important immuno-regulatory molecules such as MHC class I and CD1d, we hypothesize that it may play a role in immune responses such as cows' milk allergies and certain autoimmune diseases (e.g. insulin dependent diabetes mellitus, which has been associated with cows' milk consumption). Given this hypothesis, it would be useful to know if the conformation and biological activity of Bovß2-M could be destroyed by simple physical treatments such as heating.

Most commercially available dairy products are processed before consumption by the general public; milk, cheese and yogurt in particular are heated before packaging or fermentation. Pasteurization (72°C x 15 s) is designed primarily to kill micro-organisms present in the milk, but it also denatures and therefore destroys the functional activity of many of the milk proteins. Bovine IgG from milk is known to be largely unaffected by the

pasteurization process (87), suggesting that the same would apply to the structurally similar Bovß2-M.

We designed an assay to detect conformationally intact and biologically active Bovß2-M based on its known ability to displace the endogenous murine ß2-M and heterodimerize with the murine class I molecule D^b; to detect this event we used a unique McAb (W6-32) which only recognizes the resulting xenogeneic mouse/bovine heterodimer(23). Using this assay we showed that conditions similar to pasteurization have no effect on the biological activity of purified Bovß2-M or on Bovß2-M present in milk. We also found that even under extreme temperature conditions Bovß2-M from pasteurized milk is still able to retain much of it's biological activity, putting into question any feasible heat-based commercial method for destroying the biological activity of Bovß2-M. Finally, using traditional Western blots and polyclonal rabbit anti-Bovß2-M antiserum, we were able to show that there are large amounts of intact Bovß2-M in virtually all dairy products, including those which undergo limited fermentation or microbial culture, such as cheese or yogurt.

C. Materials and methods

Bovine ß2-M and EL4 cell line

Bovß2-M was purified from bovine colostrum via the method of Groves and Greenberg (86). The mouse thymoma cell line EL4 (K^b, D^b) was obtained from ATCC and grown in protein free hybridoma media II (PFHM-II; GIBCO/BRL Life Sciences, Gaithersburg, MD) without any fetal calf serum.

Incubation of cells with milk or purified Bovß2-M

Pasteurized homogenized whole milk (100 ml; Safeway's Lucerne Brand) was centrifuged at 400,000 x G (Beckman Ultracentrifuge L8-80) for 2h at 4°C. The central liquid supernatant was removed from the lipid (upper) and debris (lower) layers and placed into fresh 50 ml Falcon tubes. The milk supernatant was then filtered progressively through 0.8 µM, 0.4 µM and 0.22 µM MicroStar® filters (Costar, Cambridge, MA). For heating, 1 ml aliquots of filtered milk supernatant or purified Bovß2-M dissolved in PFHM-II (10µg/ml) were placed in eppendorf tubes on a heating block at 90°C. After heating for the times indicated, tubes were placed on ice to cool. EL4 incubation media was prepared by placing the 1 ml aliquots of heated milk supernatant or Bovß2-M into 9 ml of PFHM-II. This was added to $1x10^7$ EL4 cells (drained pellet following centrifugation), and incubations continued for 4 hours at 37°C / 5% CO_2. Cells were then centrifuged and washed twice in 10 ml PFHM-II each time.

Cell staining and flow cytometry analysis

After incubation with milk or Bovß2-M and washing, final drained EL4 pellets were stained by resuspending in a phosphate buffered saline (PBS) solution containing FITC conjugated purified monoclonal antibodies (1 µg/ml). The antibodies were prepared from hybridoma supernatants grown in vitro, purified by affinity chromatography over protein A sepharose, and conjugated using standard methods. Monoclonal antibodies used, together with their specificity's, were as follows: CAB-297, specific for Bovß2-M (free or cell surface associated; does not see mouse, human, or rat ß2-M); W6-32, specific for any

human MHC class I or for murine D^b paired with human or bovine ß2M; L243, specific for any HLA-DR (represents isotype control). After incubation FITC conjugated antibodies were removed by underlaying the cell/antibody suspension with 3 ml 3% w/v Ficoll/PBS, and centrifuging at 500 x G, 4°C for 5 min. Cells were analyzed on FACScan® (Beckton Dickenson Inc., Mountain View, CA).

SDS-PAGE and Western blots

All SDS-PAGE gels were cast and run in the BioRad® (Hercules, CA) Mini-PROTEAN II apparatus, using a 5% Tris/Glycine/acrylamide stacking gel and a 15% separating gel. Various commercially available food samples (≈20µl vol) were heated at 95°C for 10 min with 20µl of 7% SDS / ß-mercaptoethanol denaturing buffer. Samples were loaded and run on duplicate gels at constant current (25mA/gel) for 50-60 min. One gel was then fixed and stained for 15 min in a solution of 50% methanol, 10% acetic acid, 0.5% comassie blue G250; and destained for 60 min in 10% methanol, 10% acetic acid prior to visualization of protein bands.

The remaining duplicate gel was soaked in transfer solution (15%MeOH, Tris/Glycine buffer) for 10 min then transferred to nitocellulose at 200mA for 1 hour using a mini trans-blot apparatus (BioRad, Hercules, CA). The nitrocellulose blot was blocked with 3% PBST (3% v/v Tween-20 in PBS) for 1 hour at 4°C. The blot was incubated overnight at 4°C in 10 ml PBS pH 6.8 containing 1:1000 of the primary antibody (polyclonal rabbit anti-Bovß2-M, serum KF59, a kind gift from M. Groves). The nitrocellulose was then washed three times, using 10 ml 1% PBST for 10 min each time. The secondary antibody, alkaline phosphatase conjugated affinity purified goat anti-rabbit IgG (Jackson Immunochemicals) was added at 1:5000 in PBS pH 6.8 and incubated 1 hour at 4°C. The blot was washed as above, and finally color developed with NBT/BCIT.

D. Results

Conformationally intact, biologically active Bovß2-M is present in commercial cows' milk

As seen in figure 5.1, when EL4 cells were incubated with either 10% milk supernatant or with 1µg/ml purified Bovß2-M and then stained with the monoclonal CAB-297, identical intensity of staining (shifted considerably from cells with medium only) was seen for each group of cells. Similar results were obtained using the monoclonal W6-32 (figure 5.2). This shows that commercially available homogenized and pasteurized milk contains intact, native Bovß2-M which still possesses a conformational epitope recognized by CAB-297, and moreover, that the Bovß2-M in milk can heterodimerize with the D^b heavy chain on EL4 cells in the same way as native purified Bovß2-M.

Heat treatment reduces but does not eliminate conformationally intact Bovß2-M

Significant but not complete loss of CAB-297 staining was observed on EL4 cells incubated in 10% milk supernatant that had been heated at 90°C for 10 min (figure 5.3). A proportionately decreasing level of CAB-297 staining was seen for EL4 cells incubated in milk that had been heated at 90°C for increasing times ranging from 30-120 min (figure 5.3). Mirroring the decrease in conformationally intact Bovß2-M bound to the surface of EL4 cells was the loss of D^b associated Bovß2-M protein (i.e. decreased W6-32 staining), as shown in figure 5.4. Heating at lower temperatures, or use of shorter heating times (up to 7 min at 90°C) had no effect on reducing W6-32 staining (data not shown). A similar decrease in staining by both monoclonal antibodies was seen when EL4 cells were incubated in beta-mercaptoethanol reduced samples of milk or pure Bovß2-M (data not shown). With increasingly long periods of heat treatment, the rate at which the conformationally intact Bovß2-M disappears begins to decrease (at least as assessed by binding to/staining EL4 cells). Due to this plateau effect, significant staining by both monoclonal antibodies could be seen even after the milk supernatant had been heated at 90°C for 120 min (figures 5.3 and 5.4).

The increase in W6-32 staining intensity of the EL4 cells incubated in untreated milk supernatant or in purified Bovß2-M is less than the increase seen with CAB-297, highly suggestive that other cell surface molecules in addition to class I MHC are involved in Bovß2-M binding to EL4 cells. The relative loss of staining of the EL4 cells after heat treatment of the milk supernatant is also greater for W6-32. However, the 'plateau' in the decrease of staining for increased heating times for W6-32 is also much more pronounced than for CAB-297, with no real change in staining with milk supernatant heated at 90°C from anywhere between 30 and 120 min (figure 5.4 and data not shown). Incubation of EL4 cells in heat treated pure Bovß2-M showed the identical loss of CAB-297 staining (figure 5.5; only the 120 min data is shown). Surprisingly the W6-32 staining of EL4 cells incubated in purified Bovß2-M which was heated at 90°C for 120 min and then cooled on ice is completely unaffected compared to the unheated protein (figure 5.6). This suggests that pure Bovß2-M may behave somewhat differently upon heating/cooling than when it is present in a complex mixture of milk proteins.

Intact Bovß2-M is present in many dairy products

Intact Bovß2-M was seen in various dairy products that have undergone different forms of processing, as shown in figure 5.7. In a series of Western blots (not all shown), large quantities of intact Bovß2-M were seen in pasteurized (72°C x 15 sec.) homogenized whole milk and skim milk. Ultra high temperature (UHT) milk (125°C, 5 sec) also contains intact Bovß2-M. A variety of other foods also contain intact Bovß2-M, including yogurt, 2 different cheeses, processed cheese spread, ice cream, whey powder, etc. In comparison, raw beef contained much lower levels of Bovß2-M, and levels were even lower in cooked beef. Of interest (data not shown), the infant formula similac and similac LF contained moderate quantities of intact Bovß2-M, whereas the hydrolyzed formulas nutramigen, progestamil, alimentum, as well as isomil (soy based) did not.

E. Discussion

We have found that intact Bovine ß2-microglobulin is present in high levels of almost all commercially available pasteurized and processed dairy products, as well to a much lesser degree in raw and cooked beef. We have also found that the Bovß2-M in pasteurized/homogenized milk has the identical biological activity to that of Bovß2-M purified from untreated bovine colostrum. It is unlikely that any currently existing pasteurization process would affect the Bovß2-M content of milk significantly, as heat treatments much harsher than those typically used in commercial dairy processes (72°C x15 sec) did not cause any change in biological activity. If only minor losses of Bovß2-M result from pasteurization then individuals consuming 1 liter of milk a day would consume ≈30mg of Bovß2-M. Ingestion of large amounts of a biologically active protein such as this may potentially affect immune, allergic, or autoimmune responses in genetically susceptible individuals. ß2-microglobulin is known to act as an adjuvant to stimulate CTL mediated responses against peptides in mice(30,33), and a similar mechanism may potentially stimulate immune responses towards dairy protein antigens in general. Clearly a variety of these food antigens are known to be highly antigenic, and they frequently cause allergies in susceptible individuals. In terms of autoimmune diseases, several reports indicate a correlation between cows' milk consumption and the incidence of Type I, insulin dependent diabetes mellitus (IDDM) (82,83).

With the sensitive assays described above, we are able to easily follow the effect of various treatments on the biological activity of Bovß2-M in milk. Positive staining of the EL4 cells with the CAB-297 monoclonal is an indication of the retention of at least part of the Bovß2-M molecule's native conformation, as the CAB-297 McAb is unable to recognize reduced/denatured Bovß2-M (unpublished observations). The ability of the Bovß2-M to form a heterodimer with D^b forms an even stricter standard for assessing the native conformation and 'biological activity' of any given Bovß2-M preparation, either *in vitro* or *in vivo*. By utilizing a fortuitous cross reaction of the anti-human class I

monoclonal antibody W6-32 with the D^b/Bovß2-M heterodimer, we were able develop a method of determining function based on physical interactions with another protein, and not merely judge the activity of Bovß2-M based on the size of the protein or on the retention of a single conformational epitope seen by a specific monoclonal antibody (e.g. CAB-297). It is highly unlikely that a partially denatured or otherwise altered Bovß2-M would be able to dimerize with D^b in such a way as to generate the W6-32 epitope.

CAB-297 and W6-32 staining of EL4 cells incubated with heat treated purified Bovß2-M indicates that there is a threshold level of exogenous Bovß2-M above which all potential D^b molecules on EL4 cells have Bovß2-M associated. Heating the purified protein for increasing times presumably increases the quantity of denatured, 'conformationally incorrect' Bovß2-M present; however with the concentration of Bovß2-M used in this study (1µg/ml) there is apparently always enough biologically active/conformationally correct protein to bind to all available D^b molecules. Presumably with lower Bovß2-M concentrations or with harsher treatments we would eventually see the loss/decrease of the D^b/Bovß2-M heterodimer on the cell surface, although the capacity of the denatured Bovß2-M to re-fold on ice, particularly if the intrachain disulfide remains intact, cannot be discounted.

The level of CAB-297 staining seen in figure 5.1 indicates that there are much greater quantities of Bovß2-M bound to the cell surface of EL4 cells than can be accounted for by simple pairing with the D^b molecule. These interactions could involve other class I-like molecules such as CD1 and TL, which may have a lower affinity for Bovß2-M than does the D^b molecule, and/or they may involve some form of non-specific binding to the EL4 cell surface. CAB-297 staining also shows that Bovß2-M binding to the cell surface is greatly affected by the extended heat treatment (90°C x 120 min), both in milk and with the purified Bovß2-M solutions. However, staining occurs at about 30% of maximum even after the extended heat treatment. In contrast, heating of milk for only 30 min at 90°C drops the amount of D^b/Bovß2-M heterodimer on the cell surface by 80%. It is not clear why

there would be such a difference between the heating of milk and heating purified Bovß2-M solutions, but one likely explanation is that in the case of milk, other denatured milk proteins are interacting with Bovß2-M to sequester it and/or prevent its re-folding upon cooling.

It appears that heat treatment of cow's milk as a method of reducing the biological activity of the Bovß2-M may not be practically possible. Heating milk for longer than 7 min at 90°C is required to significantly reduce the ability of Bovß2-M to heterodimerize with murine class I. Bovß2-M is known to be able to heterodimerize in a similar fashion with human class I. Unfortunately a reagent such as W6-32, which could differentiate between a human class I/human ß2-M heterodimer and a human class I/ Bovß2-M heterodimer, does not exist, so the effects of pasteurization on the ability of Bovß2-M to interact with human class I cannot be measured directly at present. As in the mouse, it appears as there is variation in human class I/ ß2-M stability for various class I alleles present in the human population, and therefore it is likely some human class I molecules might have a high affinity for Bovß2-M (similar to D^b for the mouse), in which case the rationale for removing all biologically active Bovß2-M would still stand in the human context.

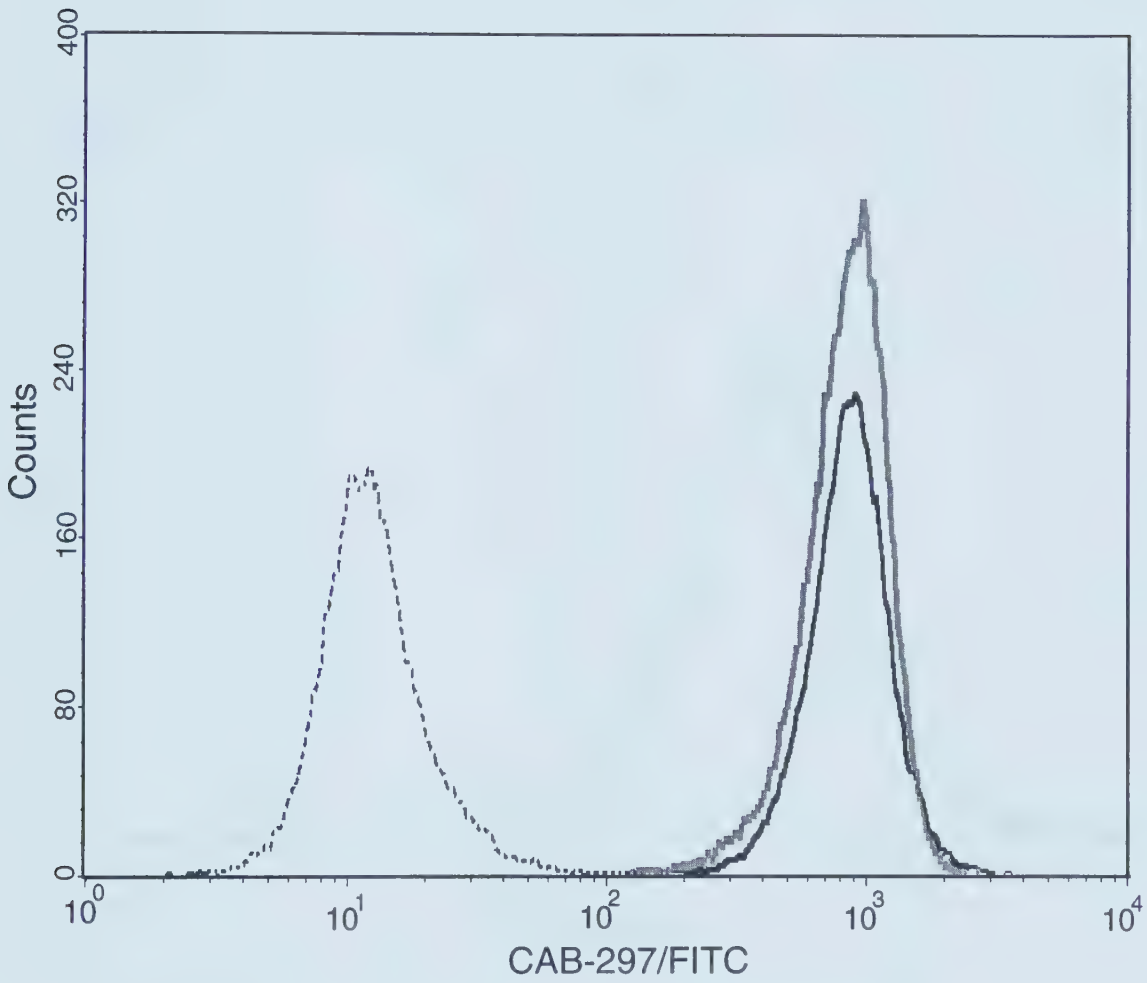


Figure 5.1. FACS profile of EL4 cells showing identical CAB-297 staining (sees a conformational epitope of Bovß2-M) after incubation with 10% homogenized milk supernatant or with purified Bovß2-M. Incubations were carried out in protein free hybridoma media (PFHM-II) for 4 hrs at 37°C. Cells were incubated in PFHM-II alone ▪▪▪▪ , PFHM-II+10% homogenized/ pasteurized milk supernatant ▬ or PFHM + purified Bovß2-M (1µg/mL) ▬ .

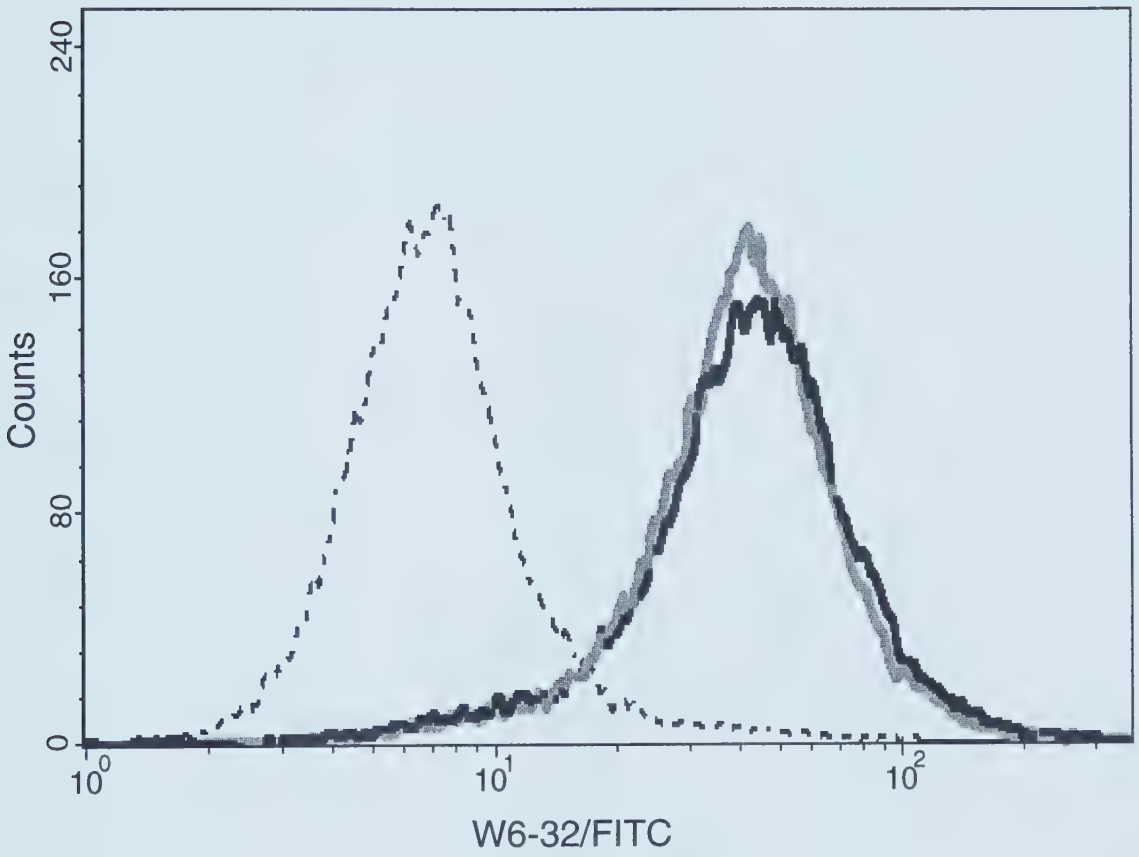


Figure 5.2. FACS profile showing identical W6-32 staining (specific for the mouse D^b/Bovß2-M heterodimer) after incubation of EL4 cells with 10% homogenized milk supernatant or purified Bovß2-M in PFHM-II for 4hrs at 37°C. Cells were incubated in PFHM-II ▪▪▪▪ , 10% homogenized milk supernatant in PFHM-II ▬ , or purified Bovß2-M (1ug/mL) in PFHM-II ▬ .

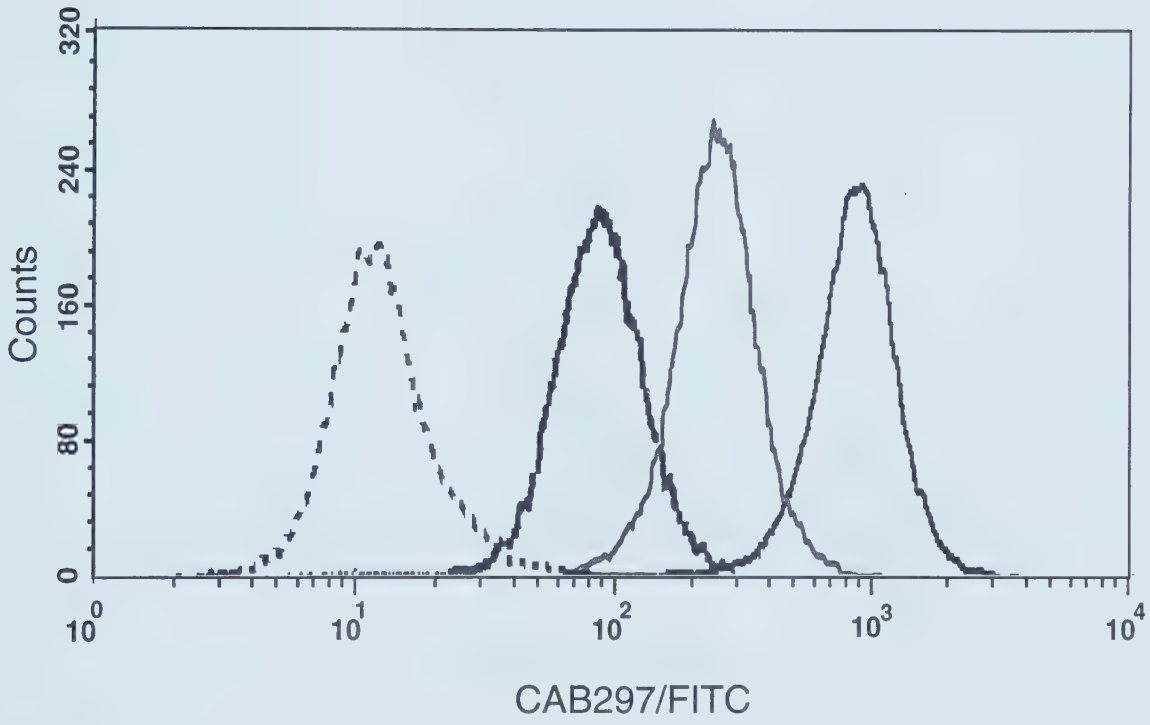


Fig 5.3. FACS profile showing effect of heating on the ability of Bovß2-M in homogenized milk supernatant to bind EL4 cells and be recognized by a conformation sensitive anti-Bovß2-M specific McAb. CAB-297 staining of EL4 cells incubated in PFHM-II for 4 hrs ▪ ▪ ▪ ▪ , with 10% homogenized milk supernatant ▬, same supernatant heated 90°C /10 min ▬, same supernatant heated 90°C/120 min ▬.

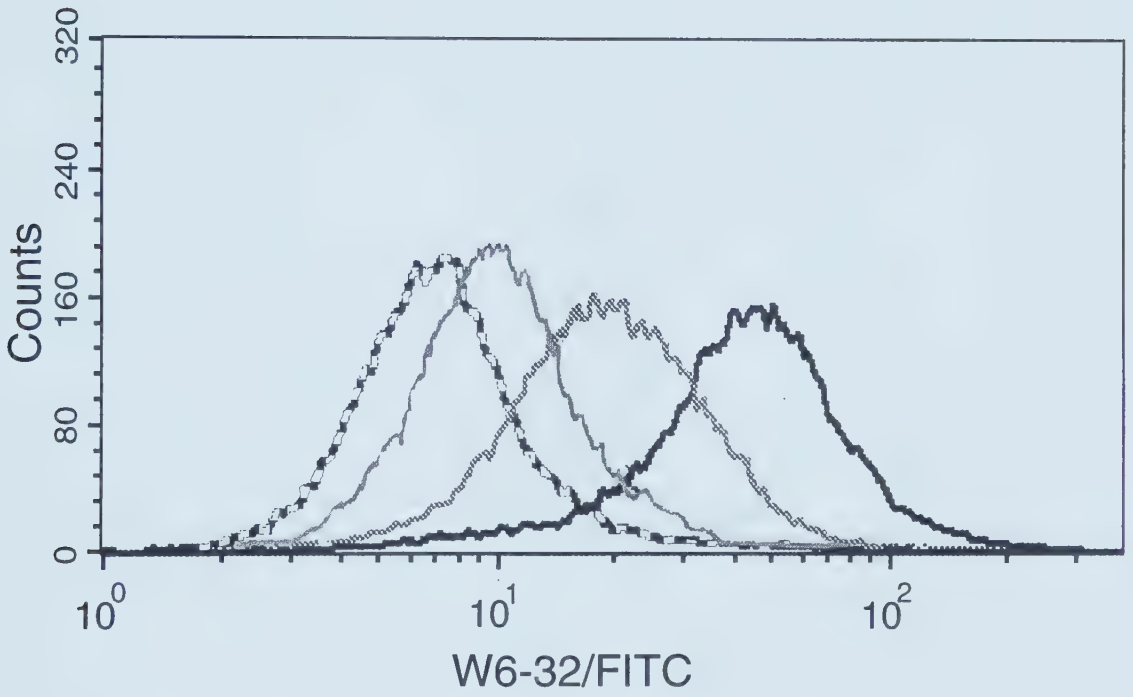


Figure 5.4. FACS profile showing the effects of heating on the ability of Bovß2-M in homogenized milk supernatant to heterodimerize with the murine D^b class I molecule. W6-32 staining of EL4 cells incubated in PFHM-II for 4 hrs ▪ ▪▪ ▪ , with 10% homogenized milk supernatant ▬▬ , same supernatant heated 90°C /10 min ▒▒▒ , same supernatant heated 90°C/120 min ▬▬ .

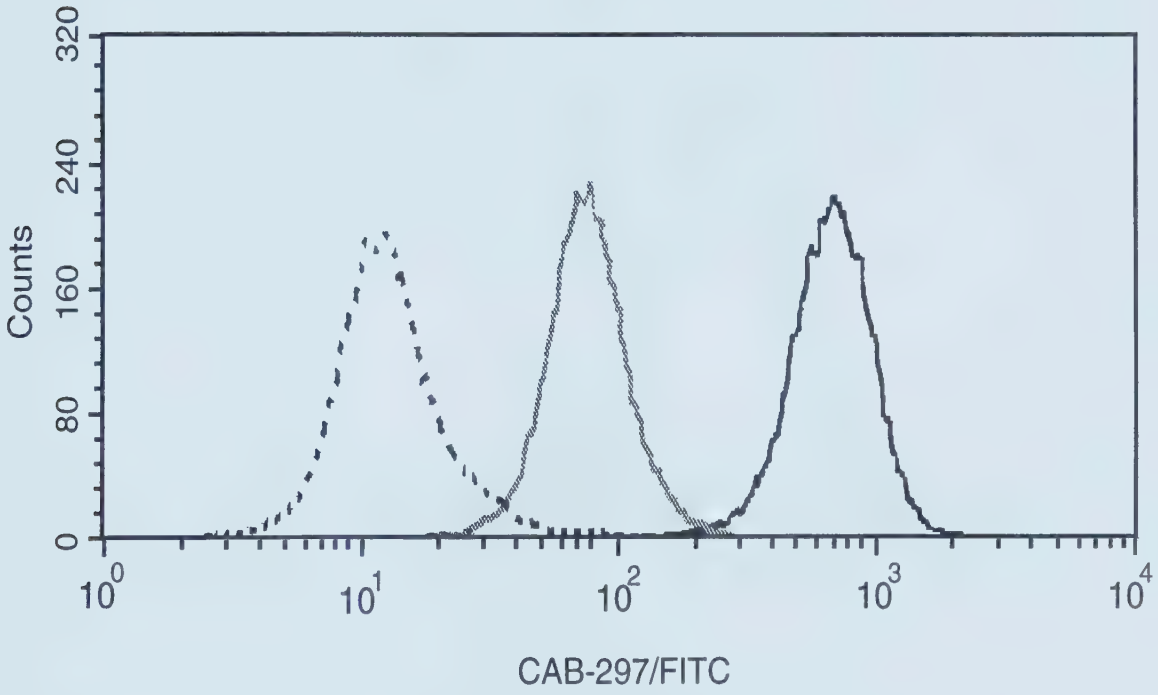


Figure 5.5. FACS profile showing the effects of heating on ability of purified Bovß2-M to bind EL4 cells and be recognized by a conformation sensitive anti-Bovß2-M McAb. CAB-297 staining of EL4 cells incubated in PFHM-II for 4 hrs ▪▪▪▪, PFHM-II + Bovß2-M (1µg/mL) ▬▬ , PFHM-II + Bovß2-M (1µg/mL) heated 90°C for 120 min and cooled on ice before addition to media ▬▬ .

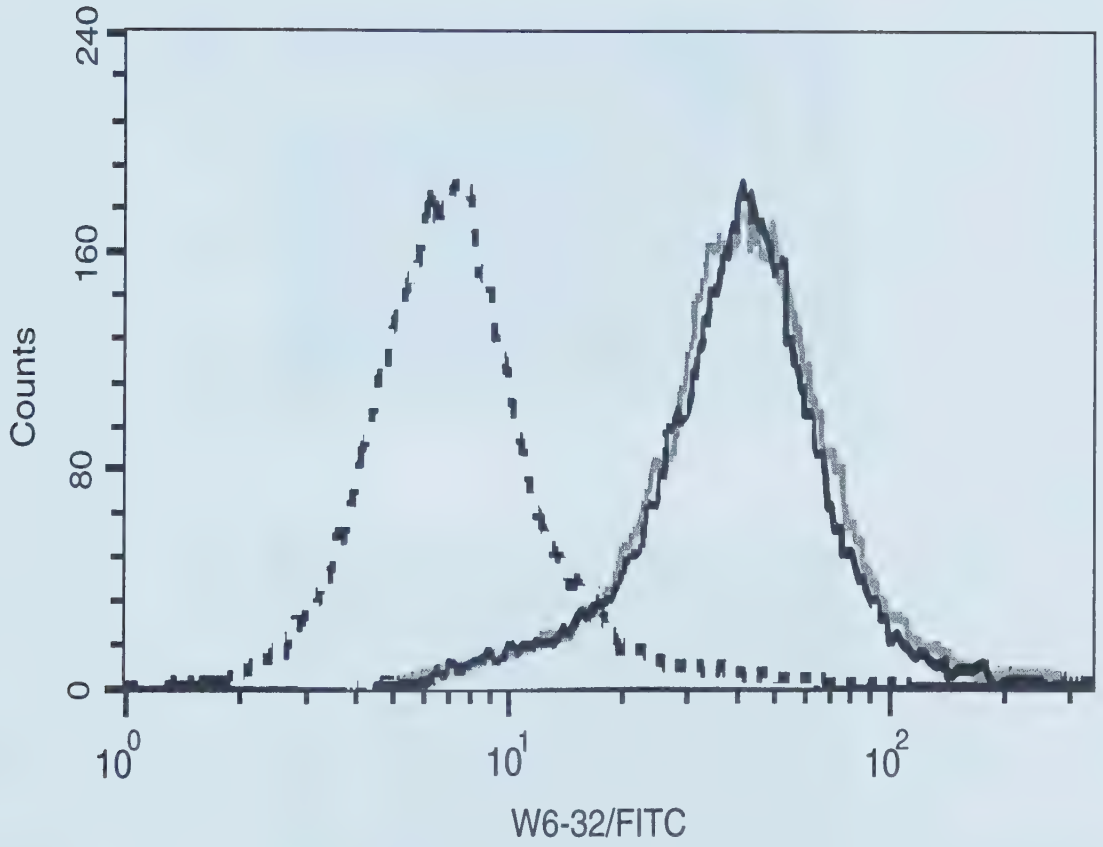


Fig 5.6. FACS profile showing the effects of heating on the ability of purified Bovß2-M to heterodimerize with murine class I D^b molecule. W6-32 staining of EL4 cells incubated in PFHM-II for 4hrs ▪▪▪▪ , PFHM-II + Bovß2-M (1µg/mL) ▬▬ , PFHM-II + Bovß2-M (1µg/mL) heated 90°C for 120 min and cooled on ice before addition to media ▬▬ .

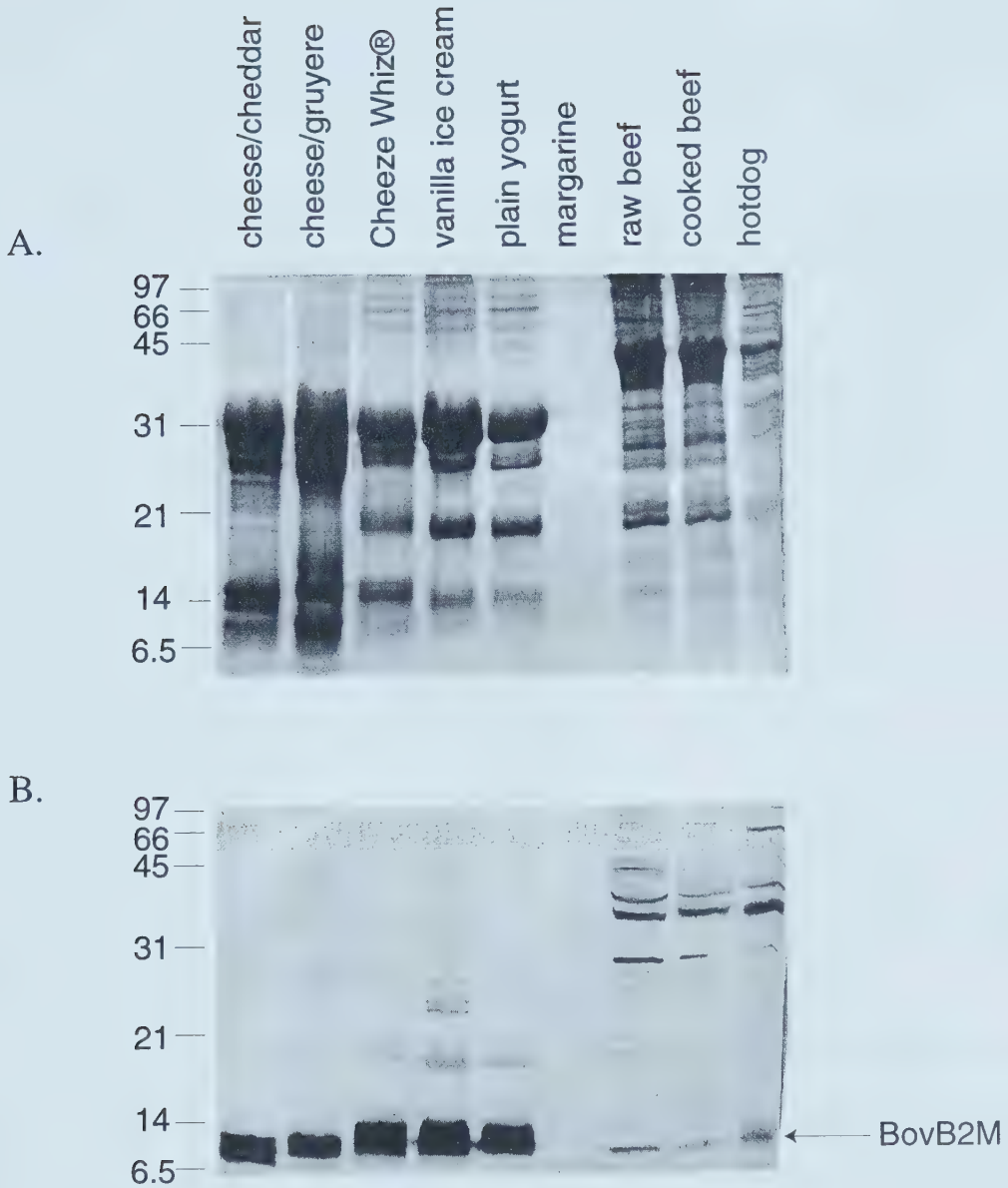


Figure 5.7. A) SDS-PAGE gel stained with comassie blue and B) Western blot from an identical gel probed with rabbit polyclonal antisera against Bovß2-M (KF59). A variety of commercially available food products containing Bovß2-M were analyzed.

CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

In any exploratory study such as this, it is difficult not to be overwhelmed with the varied potential future directions which our research could take. Considering that high levels of bovine IgG are found in milk, it is not surprising that we found similar high levels of Bovß2-M present in commercially available dairy products. However, it was of considerable interest to find that the protein retained much of it's biological activity after heat treatments which were much 'harsher' than any used in commercial processes. Presumably any dairy consuming individual introduces large quantities of fully active Bovß2-M into the small intestine, having unknown effects on health and immune function. Perhaps the most surprising observation was that of the specific transport of low levels of intact and biologically active Bovß2-M into the circulation of adult mice after they voluntarily ingested the protein. At this point the transport mechanism is unknown, but further histology of intestinal wall tissues, together with specific transport studies using radiolabeled protein would be expected to shed light on this question.

It is not yet clear how the passage of small quantities of ingested Bovß2-M into the circulation might be related to the pathogenesis of IDDM. However, in general terms the presence of a foreign protein capable of interacting with and altering class I presentation has the potential to 'unbalance' the cytolytic response, a response which is known to play an integral role in the development of the disease. The levels of Bovß2M observed free in circulation and associated with various murine class I and class I like proteins are very low. At this point we have not determined if other organs may also be sites for heavy deposition of Bovß2M. It seems likely that after 48 h the high levels of radioactivity still present in ^{125}I-Bovß2M fed mice would be the result of protein associated counts, as the majority of free ^{125}I would have been filtered out of the circulation by the kidneys. However

comparable ^{125}I-Bovß2M counts from the organs isolated (spleen, SI) determined from immunoprecipitations were not found. It is possible that our method of extracting Bovß2M via immunoprecipitations is unable to detect the protein bound to other plasma or cellular components. Traditional methods of TCA precipitation might aid in determining if Bovß2M binding components exists.

From our initial plasma half lives experiments, we have discovered interesting differences between the MHC matched strains NOD and NOR. Further metabolic studies of this nature, studying Bovß2-M levels in urine and extra vascular spaces, would indicate if in fact the differences in plasma half lives was a result of physiological differences or of induced immunological differences. In vitro stimulation of lymphocytes from both strains with Bovß2-M would determine if the NOD strain was able to mount a greater response towards the protein than the NOR strain. Inclusion of mice that are in the process of becoming diabetic would be of interest in determining if elevated levels of cross-reactive antibodies play a role in the progression to diabetes. The ability of Bovß2M to bind to class I present on RBCs may also play a role in the differential, apparent loss of Bovß2M in murine plasma. We were unable to determine the extent of this potential interaction as certain erythrocyte proteins such as a-gamma globulins preferentially precipitate without specific Bovß2M McAbs (data not shown). Initially, chromatography of a mixture of mouse serum with Bovß2M would allow identification of potential binding factors. Combining these experiments with more comprehensive metabolic studies where isolation of protein from whole individual organs are performed would afford us a clear picture of where Bovß2M, once in circulation, resides until it is catabolized.

Results from any of the future studies of Bovß2M interactions with human class I molecules might allow insights into weak associations of class I haplotypes with IDDM incidence. It is conceivable that haplotype that have been found linked with IDDM could be less stable with human ß2M and more likely to be replaced with Bovß2M. The resulting xenogeneic heterodimers would presumably contain unique epitopes or have altered peptide

binding attributes that might trigger an autoimmune response. Development of McAbs specific for human class I with Bovß2M bound (similar to McAb W6-32 for murine D^b + Bovß2-M) would be incredibly useful in further studies, and may warrant development. The production of these could be accomplished by immunization of mice with purified human class I heavy chain paired with Bovß2-M, both which are currently at our disposal.

Unfortunately human studies using radiolabeled Bovß2-M to study oral uptake and absorption will be hindered by the fact that Bovß2M appears to be deposited in high concentrations, and in a relatively stable configuration, on the inner intestinal wall of mice and rats (unpublished observations). Thus radioactively labeled protein probably cannot be used in human studies due to potential deleterious effects on the radiosensitive gut lining. Other methods to follow Bovß2-M passage in humans could include detection of protein labeled by some other means, or by analyzing human serum and/or urine using a Bovß2-M specific ELISA such as that used to measure human ß2M levels. These methods in our hands suffer from many cross reactions and non-specific effects which interfere with the accurate determination of any possible increased levels of Bovß2M after cows' milk consumption. Possibly use of novel combinations of specific anti Bovß2-M McAbs at our disposal in competitive radioactive ELISAs may provide a method for following the kinetics of appearance/disappearance of systemic Bovß2M in humans consuming dairy products. These assays could be extended to human breast milk, where similar methods have been used to detect bovine milk proteins in human colostrum after ingestion of dairy products (107). At this point we have no reason to believe that detectable levels of Bovß2-M will not be found in human colostrum in mothers who consume dairy products, in a similar fashion to the closely related bovine IgG.

Further studies of Bovß2M interaction with class I like molecules could shed more light on the potential involvement of Bovß2M in the development of immune functions in the infant, arguably the most important period in the development of the immune system. Of particular interest is the FcRn receptor which, although apparently designed to transport

maternal IgG (and perhaps maternal ß2-M), offers an attractive mechanism of co-transport of dietary Bovß2M and dietary bovine IgG. Our results indicate that the homologous low affinity FcRgII/III receptors bind low levels of Bovß2M, but clearly similar studies need to be performed with specific FcRn monoclonal antibodies. In any case, the role of FcRn in transporting large amounts of Bovß2M into the fetal and neonatal circulation cannot be discounted.

Exhaustive studies of the various human class I haplotype stabilities, determined by their relative ability to associate with Bovß2-M in the presence of free human ß2-M, would be a reasonable future step if transfer to human circulation is observed. If significant levels do not appear in human circulation, differences in the strength of Bovß2M associations with class I could still prove useful in determining relative stability and thus the ability to become unfolded and to rebind exogenous peptide. A strategy such as this might provide interesting insights into class I autoimmune disease associations even if ß2-M independent. It is not clear what the future of Bovß2M is in the field of IDDM; in fact it may not play any significant role in the disease. However it has already shown itself to be a useful tool for determining class I heterodimer stability, and for detecting immune activation in vivo. Further use of labeled Bovß2M in histology (such as in Chapter 3) of inflamed areas will allow greater pinpointing of the function behind cytolytic processes in autoimmune and other diseases.

Future studies of Bovß2M must include human models to determine the relevance of any of our observations to correlations of cows' milk consumption and IDDM incidence. If significant levels of dietary Bovß2M can pass through the small intestine wall and into circulation in humans, it will be important to determine the potential consequences of such an occurrence. While it appears that little can be done to completely remove the biological activity of dietary Bovß2M, other strategies such as acid or proteolytic hydrolysis might prove useful.

Two alleles of Bovß2-M have been found in different populations of cattle, which result in an amino acid difference in the protein(109) (see also Table 1.1). Reports have also been made of populations such as the Masai tribes of Africa, and the population of Iceland, which consume very large quantities of cows' milk but which have very low incidence of IDDM (R. Elliott, personal communication). It is possible that the amino acid variation between these two Bovß2-M alleles may play a role in the avidity of Bovß2-M for human class I, with the Masai and Icelandic cattle displaying an allele less able to bind human class I, and therefore less likely to promote an autoreactive cytolytic response. It would be interesting to obtain milk and DNA samples from both these two unique cattle populations to test this hypothesis. If the amino acid variation of these alleles did play a role in a differential exchange of Bovß2-M for human ß2-M on human class I, a genetic approach to continuing dairy consumption without the associated risk of IDDM would be possible.

Finally, the relevance of oral tolerance for controlling mice and rats immune response to various dietary antigens has become a contentious issue. While this effect has been shown to occur in mice and rats, it is strange that attempts to orally tolerize humans in the same manner have been largely unsuccessful. Based on our observations in chapter 2, it is most likely that the animals being fed by gavage would at some point receive relatively large amounts of proteins or peptides directly into the lungs. Due to the natural function of the alveolar macrophages, these proteins could easily be processed and have unknown affects on immune stimulation or suppression. It would be of considerable interest to attempt to induce tolerance of mice to antigen via low levels of isotonic protein solutions applied to the lungs. Recent studies concerning the viability of inhaled insulin treatments for controlling diabetic glucose levels suggest that proteins can be applied to the lungs without adverse affects. If tolerance could be achieved via this method, novel treatments of high risk individuals could be attempted.

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